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HRas intracellular trafficking and signal transduction

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HRas intracellular trafficking and signal transduction

by

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A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Genetics

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2007

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All men dream but not equally. Those who dream by night in the dusty recesses of their minds wake in the day to find that it was vanity; but the dreamers of the day are dangerous men, for they may act their dreams with open eyes, to make it possible.

T. E. Lawrence, *Seven Pillars of Wisdom, a Triumph*

Table of Contents

List of Figures.....	viii
List of Tables	x
Abstract.....	xi
Chapter 1 General Introduction.....	1
Molecular responses	2
Cellular responses	3
Thesis Organization	5
<i>HRas does not need COP I- or COP II-dependent vesicular transport to reach</i> <i>the plasma membrane</i>	<i>5</i>
<i>Golgi-independent trafficking requires the conservation of HRas hypervariable</i> <i>region</i>	<i>6</i>
<i>Internalization of HRas via Arf6-associated endosomal pathway.....</i>	<i>6</i>
Chapter 5 General conclusions	7
General Literature Review	7
Posttranslational modification	8
Ras signal transduction	9
Ras mediated signal transduction from endomembranes	10
Vesicular endocytosis	11
Phagocytosis	12
Pinocytosis	12
Endosomal Recycling Center.....	13
Vesicular exocytosis	14
Cargo transport from ER to Golgi	14
Cargo transport from Golgi to ER	15
Microtubules and intracellular trafficking	16
Concluding Remarks.....	17
References.....	17
Figure Legends.....	28

Figures.....	31
H-Ras does not need COP I- or COP II-dependent vesicular transport to reach the plasma membrane.....	37
Abstract	37
Introduction.....	38
Materials and Methods.....	41
Mutants, Plasmids and Antibodies.....	41
Cell Culture and transfection	41
Counting cells	41
Brefeldin A, nocodazole and cycloheximide treatment.....	42
Immuno-fluorescence imaging	42
Movies	43
Results.....	43
H-Ras proteins often show little accumulation in the Golgi.....	43
Nascent H-Ras moves much faster than YFP-GT46 to the plasma membrane	45
Neither Brefeldin A nor dominant negative Sar1 prevent H-Ras movement to the plasma membrane.....	47
Structures that carry H-Ras do not show directed movement to the plasma membrane in BFA-treated cells	50
Nocodazole does not prevent H-Ras movement to the plasma membrane.....	52
Some H-Ras accumulates at the ERGIC when the classical pathway is blocked.....	53
H-Ras that is in the ERGIC does not access the alternate pathway in nocodazole-treated cells	54
Discussion	56
Acknowledgements.....	59
References.....	59
Figure Legends.....	61
Table Legends.....	64
Figures.....	66
Trafficking requires conservation of the HRas hypervariable region.....	74

Abstract	74
Introduction	75
Materials and Methods	77
DNA and plasmids	77
Cell culture and transfection	77
Cell counts	78
Brefeldin A, Nocodazole, and Cycloheximide treatments	78
Immunofluorescence	79
Microscopy	80
Results	80
GFP-HRas expression in multiple cell types	80
HRas plasma membrane localization with BFA	83
Mono-palmitoylated HRas does not accumulate in the Golgi	84
Palmitoylation requirements for Golgi-independent trafficking	84
Non-palmitoylated residues and HRas plasma membrane localization	86
Conservation of the amino acids further upstream of the CAAX box is also required for Golgi-independent HRas trafficking	87
Discussion	88
Golgi-independent trafficking exists in multiple cell types	88
Palmitoylation is required for Golgi-independent trafficking mechanism	88
Residues between the lipid moieties are important in HRas trafficking	89
Conservation of HRas hypervariable region and Golgi-independent trafficking	89
Concluding remarks	90
Acknowledgements	91
References	91
Figure Legends	94
Table Legends	98
Figures	99
Both active and inactive forms of H-Ras bind to clathrin-independent endosomes associated with ARF6	110

Abstract	110
Introduction	111
Materials and methods	113
Plasmids, cell culture, and DNA transfection	113
Antibodies	114
Live cell labeling with transferrin or MHC-I antibody	115
Immuno-isolation of membranes	116
Immunoblotting	117
Immunofluorescence and preparation of sonicated membrane fragments	117
Results	118
H-Ras does not utilize the clathrin-dependent pathway	118
Endosomes associated with Arf6 bind H-Ras	120
EGF and H-Ras activation on Arf6 endosomes	123
Very little Raf-1 binds to H-Ras61L on Arf6 endosomes	125
H-Ras is present in the perinuclear endosomal recycling center	125
KRas4B and ExtRas binding to Arf6 endosomes differs from H-Ras	127
The C-terminal domain of H-Ras or KRas4B proteins can target GFP to the Arf6 endocytic pathway	128
Discussion	128
Properties that influence binding of Ras proteins to Arf6-associated vesicles	129
The role of endosomes in H-Ras signaling	130
Acknowledgements	131
References	131
Figure Legends	137
Figures	143
Chapter 5 General Conclusions	150
Ras posttranslational modifications	150
Ras intracellular trafficking	151
Summary of the Studies	152
<i>HRas does not need COP I- or COP II-dependent vesicular transport to reach the</i>	

<i>plasma membrane</i>	152
<i>Golgi-independent trafficking requires conservation of the HRas hypervariable</i> <i>region</i>	154
<i>Internalization of HRas via ARF6-associated endosomal pathway</i>	155
Biological Implications	156
Suggestions for Future Research	157
Additional control experiments	157
Experiments that extend the research	158
References	158
Figure Legends.....	161
Figures.....	164
Appendix.....	169
Acknowledgements	170
Committee members and colleagues	170
Families and friends.....	171

List of Figures

Figure 1 Ras exocytosis	31
Figure 2 Ras domains.....	32
Figure 3 HRas C-terminus modifications	33
Figure 4 Ras-mediated MAPK signal transduction pathway.....	34
Figure 5 Endocytic vesicle trafficking	35
Figure 6 Types of endocytic vesicles.....	36
Figure 1 H-Ras is not in the perinuclear area in many cells	66
Figure 2 H-Ras moves faster than YFP-GT46 to the plasma membrane	68
Figure 3 BFA or dominant negative Sar1 do not prevent H-Ras from reaching the plasma membrane	69
Figure 4 Live cell imaging shows no obvious vesicular traffic of H-Ras to the plasma membrane when BFA is present	70
Figure 5 H-Ras still reaches the plasma membrane when nocodazole is present.....	71
Figure 6 When nocodazole or BFA is present, a portion of H-Ras accumulates in the ERGIC.....	72
Figure 7 H-Ras does not leave the ERGIC in the presence of nocodazole. COS-7 cells were transfected with H-Ras ^{Q61L} DNA.....	73
Figure 1 GFP-HRas Q61L and mGFP-HRas Q61L accumulate in the Golgi area	99
Figure 2 GFP-HRas Q61L expression in multiple cell types	100
Figure 3 GFP-HRas Q61L utilizes Golgi-independent, microtubule-independent exocytosis.....	103
Figure 4 HRas mono-palmitoylated mutants	104
Figure 5 Mono-palmitoylated HRas does not utilize Golgi-independent, microtubule- independent exocytosis	105
Figure 6 Mono-palmitoylated HRas does not retreat back into ER in the presence of BFA	106
Figure 7 HRas CDQCE mutant does not localize to the plasma membrane	107
Figure 8 HRas alanine mutants	108

Figure 9 HRas alanine mutants do not utilize the Golgi-independent, microtubule-independent exocytosis mechanism	109
Figure 1 H-Ras is absent from early endocytic vesicles of the clathrin-dependent pathway.	143
Figure 2 H-Ras is present on endosomal membranes that contain MHC-I and Arf6	144
Figure 3 EGF exposure results in GFP-RBD recruitment to enlarged endosomes in cells expressing H-RasWT and Arf6-Q67L.....	145
Figure 4 Raf-1 is present on Arf6-containing endosomes	146
Figure 5 H-Ras is present in the perinuclear endosomal recycling center.....	147
Figure 6 Endocytosis of KRas4B and ExtRas differs from H-Ras	148
Figure 7 C-terminal residues of H-Ras or KRas4B proteins can target GFP to the Arf6 endocytic pathway	149
Figure 1 Ras domains.....	164
Figure 2 Ras-mediated MAPK signal transduction pathway.....	165
Figure 3 HRas C-terminus modifications	166
Figure 4 Ras exocytosis	167
Figure 5 Ras endocytosis	168

List of Tables

Table1 HRas perinuclear distribution is distinct from the pattern of a protein in the traditional vesicular trafficking pathway	67
Table 1 HRas perinuclear distribution is distinct from the pattern of a protein in the classical vesicular trafficking pathway in NIH 3T3 and Cos-7 cells.....	101
Table 2 HRas perinuclear distribution is distinct from the pattern of a protein in the classical vesicular trafficking pathway in MDCK and HeLa cells.	102

Abstract

Cancer, which is characterized by uncontrolled cell division and proliferation, is the second leading cause of death in the United States. Of these deaths, approximately 30% are the result of mutations in Ras protein. When activated, Ras propagates multiple signal transduction cascades and induces cell proliferation, differentiation, and apoptosis. Within the Ras subfamily, there are several isoforms of Ras which differ in their posttranslational modifications. The modifications include proteolytic cleavage of the CAAX box, farnesylation, and palmitoylation which act as plasma membrane anchors where most signal transduction takes place.

This dissertation demonstrates that Ras endocytosis takes place through a novel vesicular mechanism before being recycled. On the contrary, during exocytosis, Ras does not predominantly use the vesicular trafficking to reach the plasma membrane. Finally, work presented here will show the importance of the Ras hypervariable region and how this region directs membrane localization and trafficking. Together this body of work offers valuable insight into intracellular trafficking and provides a new direction toward developing more effective cancer therapies.

Chapter 1

General Introduction

The ultimate goal behind studying Ras signal transduction is to understand Ras-mediated biological activity. Ras is a molecular switch that causes cell proliferation, differentiation, and apoptosis. [1-4] Overexpression of activated Ras frequently causes cells to ignore contact inhibition and grow in small clusters called *foci*. Formation of foci often is an indicator of cells' oncogenicity, the cells potential for becoming a type of cancer. [5-7] Mutations in Ras are very common among many types of cancers (~30% of all human cancers). More specifically, Ras mutations can be attributed to 90% of pancreatic, 35% of lung [8], and 60% of thyroid cancers. [9] Although not specifically named, many other types of cancers also have been linked to Ras mutations. [10]

As a disease, cancer is the second most common cause of death in the U.S. The American Cancer Society estimates that approximately 1.4 million new cases of cancer were diagnosed in the U.S. in 2006 alone. [11] Currently, multiple treatments for cancers are available. Some of these treatments target Ras lipid modifications and vesicular trafficking from the Golgi to the plasma membrane. [12-14] Increasing our understanding about Ras intracellular trafficking and signal transduction cascades may well lead to the development of more effective cancer treatments with minimal side effects and, ultimately, the elimination of cancer. This goal, however, cannot be accomplished without understanding cancer at the molecular and cellular levels. At a molecular level, Ras can interact with multiple downstream signal transducers and transcription factors. A mutation in Ras that causes constant activation of the downstream effectors results in constitutive activation of Ras-

mediated signal transduction cascades. These signal transduction cascades result in biological responses at the cellular level which attack cancer at the cellular level. [15, 16]

Molecular responses

Ras is a GTP binding protein. GTP binding proteins (GTPases) are members of a superfamily of proteins that can act as molecular switches. As their name indicates, the functions of GTPase proteins depend on an ability to exchange GDP (inactive) for GTP (active) and visa versa. The exchange between GTP and GDP often requires interaction with other proteins. For example, GDP Exchange Factor (GEF) is needed for GDP to GTP exchange and GAP (GTPase Activating Protein) proteins are needed for GTP hydrolysis to GDP. [17]

Ras is perhaps one of the most extensively studied members of the G-protein family and belongs to the monomeric subcategory. Much like its family members, Ras operates also by GTP/GDP exchange. Ras is universally expressed in all eukaryotes and it shares approximately 40–50% sequence homology with other family members. [18] To date, four major isoforms of Ras have been identified: HRas, KRas A, KRas B, and NRas. These isoforms are highly conserved with one another (approximately 85%) and most of the isoform variations occur at the carboxy terminus. [19]

In order for Ras to become fully functional, newly synthesized proteins must be posttranslationally modified with lipid moieties that later serve as membrane anchors. More specifically, the carboxy terminus of Ras is described as the *CAAX* box, where *C* represents cysteine, *AA* represents aliphatic amino acids, and *X* represents any amino acid. The posttranslational modification process begins when —AAX of the CAAX box is cleaved. Then a farnesyl group is added to the terminal cysteine (C) at the newly exposed carboxy

terminus. [20] In the case of KRas, this farnesyl group along with upstream poly-lysine residues are sufficient to anchor the protein to the cytoplasmic leaflet of the plasma membrane. For NRas and HRas, which lack upstream poly-lysine residues, upstream cysteines acquire additional palmitate groups—one and two groups respectively. [21]

Once securely attached to the plasma membrane, activated Ras can produce multiple responses at both the molecular and cellular levels. [2, 22-24] On a molecular level, Ras is able to interact with multiple downstream effectors and activate signal transduction cascades. Some of the more commonly studied Ras downstream effectors are: c-RAF (Receptor Associated Factor), Phosphatidylinositol-3 Kinase (PI3K) [25], and JNK. [26] At a cellular level, examples of Ras-induced responses include proliferation, differentiation, and apoptosis. Expressions of Ras in different kinds of cells result in different biological responses. For instance, when Ras is activated in a mouse fibroblast (NIH 3T3), it causes cell proliferation and, if Ras activation is prolonged, the cells lose their ability to regulate their growth by contact inhibition. [27] In contrast, if Ras is activated in neuronal cells like PC-12 (pheochromocytoma), cells begin to grow neurites and differentiate. [28]

Cellular responses

Contrary to the traditional school of thought, recent findings indicate that subcellular Ras localization is also important in Ras signal transduction cascades. [29] Ras isoforms are often retained in endomembranous structures such as the Golgi apparatus, Endoplasmic Reticulum [30], and even mitochondria. [31] Signal transduction cascades activated from these endomembranes seem to have longer lifespans than if they were activated at the plasma membrane [29, 32-34] More specifically, when KRas is activated from the mitochondria, it

stimulates apoptosis [31], whereas KRas activated at the plasma membrane results in cell proliferation and differentiation. [35]

Because proper membrane recognition and binding are vital for Ras to interact with its downstream effectors, it is not hard to imagine that Ras trafficking also plays a crucial role in Ras-mediated signal transduction and subsequent cellular response. [36] Until recently, the common understanding has been that the primary mode for Ras trafficking—or at least HRas and NRas—is through vesicles. [37-41] Vesicular trafficking begins at the Endoplasmic Reticulum (ER). Vesicles begin to form in the ER by aggregation of Coat Protein Complex II (COP II) protein complexes [42-44], while COP II complexes are activated by another small GTPase, Sec-related, Ras-related protein 1 (Sar1). [45] COP II complex aggregations recruit cargo proteins and vesicles are released into the ER Golgi Intermediate Compartment (ERGIC). Once at the ERGIC, cargo proteins and vesicles move to the Golgi. Then from the Golgi, the vesicles containing Ras travel on microtubule tracks to reach the plasma membrane. [46] (**Figure 1**) Also at the Golgi, a small fraction of proteins retreat back to ER. The reverse trafficking from Golgi to ER is mediated by Coat Protein Complex I (COP I) protein complexes. [42, 47, 48]

Once at the plasma membrane, Ras attaches to the cytoplasmic leaflet by HRas—lipid modifications—or by KRas—lipid modification and polylysine residues. [39] Membrane adhesion of Ras molecules is brief and dynamic. After activating its downstream partners, some Ras molecules are removed from the plasma membrane via endocytosis. [49] Generally, endocytosis implies an inward trafficking with aid of vesicles. To date, several different types of vesicles have been known to facilitate Ras endocytosis, but the vast majority of inward-bound Ras were thought to be located on the clathrin-coated vesicles. [50]

Recent discoveries [51, 52] suggest that it is possible for Ras to maintain its ability to signal and utilize non-clathrin vesicles to reach the Endosomal Recycling Center (ERC)—a final destination for endocytic vesicles.

Thesis Organization

This dissertation, which follows the three-article structure set out by the Iowa State University Graduate College, is organized into five chapters. This first chapter offers an overview of the basic premises of the Ras family of molecular and cellular mechanisms and provides an overview of each of the three articles submitted for publication resulting from research I conducted in Dr. Janice E. Buss's laboratory at Iowa State University between 2002 and 2007; my specific contributions to the research and the articles are also noted. A general literature review closes this chapter, and the final chapter offers general conclusions.

HRas does not need COP I- or COP II-dependent vesicular transport to reach the plasma membrane

The first manuscript, "HRas does not need COP I- or COP II-dependent vesicular transport to reach the plasma membrane," presents a new exocytosis mechanism for HRas. This manuscript, which has been accepted for publication by the *Journal of Biological Chemistry*, suggests that HRas can reach the plasma membrane by a non-vesicular, Golgi-independent mechanism.

I am the second author on this paper; Hui Zheng is the first author of this work and Dr. Buss is the third author. My specific contribution involved demonstrating that not every cell expressing HRas in a population shows Golgi accumulation and investigating how HRas moves from the ER to the plasma membrane in a microtubule-independent pathway.

Golgi-independent trafficking requires the conservation of HRas hypervariable region

The manuscript, “Golgi-independent trafficking requires the conservation of HRas hypervariable region,” will be submitted to the *Journal of Cell Biology*. This manuscript expands upon findings that were established in “HRas does not need COP I- or COP II-dependent vesicular transport to reach the plasma membrane,” the third of three articles presented in this dissertation. Specifically, I examined whether Golgi-independent trafficking of HRas was available across multiple cell types., I also investigated what role(s), if any, the HRas hypervariable region plays on trafficking and membrane localization.

I am the sole author of this paper; I conducted the research and prepared the results reported here.

Internalization of HRas via Arf6-associated endosomal pathway

In the manuscript, “Internalization of HRas via Arf6-associated endosomal pathway,” a clathrin-independent endocytosis pathway for HRas was examined. The study presents the possibility that HRas can be activated even during transit. I investigated how both HRas and KRas are removed from the plasma membrane via vesicular endocytosis. I found that HRas-containing ARF6 endosomes merge, then localize to the Endosomal Recycling Center (ERC). Finally, in this article, I demonstrate that the terminal 10 amino acids of HRas contain enough trafficking signals to move the Green Fluorescent Protein (GFP) from and to the plasma membrane and re-localize the GFP to the ERC.

Along with Xing Wang, I am the co-first author of this article. Under the supervision of Dr. Buss, I conducted a significant portion of the laboratory work, including designing and carrying out experiments, arranging for required materials, and recording the research process.

Chapter 5 General conclusions

Chapter 5 summarizes the work presented in this dissertation and reports the major insights into HRas trafficking, lipid modification, and signal transduction derived from this work. Further, this chapter discusses the biological implications of this work and offers suggestions for future research in the areas of HRas trafficking and signal transduction.

General Literature Review

Rat Sarcoma protein, Ras, is a major player in signal transduction that regulates cell proliferation, differentiation, and apoptosis. [53] In the past few decades, interest in Ras has grown among the scientists because [54] of its oncogenic ability. Although Ras is ubiquitously expressed, the isoforms differ from one another in their abilities to trigger different forms of carcinomas. For instance, KRas mutants are responsible for approximately 15–20% of small cell lung cancers and 95% of pancreatic adenocarcinomas. [54] Malignant thyroid cancers, on the other hand, could have HRas, KRas, or NRas mutations. Taken together, approximately 30% of all human cancers contain Ras gene mutations. [10] This kind of oncogenic ability in conjunction with its complex signal transduction activation capabilities makes Ras not only a great tool for better understanding tumorigenesis but also a potential candidate for cancer drug development.

Ras is a member of the small or monomeric GTPase superfamily. To date, there are approximately 150 known members in this family. [55] Like many of its fellow family members, Ras acts as a molecular switch that resonates between its active, GTP-bound form and its inactive GDP-bound form. [19] In simple terms, as a molecular switch, Ras acts to either turn on or off cell divisions. When activated by its upstream signal transducer, Guanine nucleotide Exchange Factor (GEF), Ras is recruited to a membranous platform where it

releases the GDP and binds a GTP in its switch regions. [18] This exchange then causes a conformational change that will allow activation of downstream effectors.

In humans, there are four major isoforms of Ras: HRas, KRas 4A, KRas 4B, and NRas. Alternative splicing of the fourth exon results in the two KRas isoforms, KRas 4A and KRas 4B. [39] Conservation and sequence identity among these four isoforms of Ras is very high—about 85% within the first 165 residues at the N-terminus—while the remaining 20-odd amino acids at the C-terminus vary greatly from isoform to isoform. [55] **Figure 2** shows switch regions, domains, and Ras C-terminus variations between isoforms.

Posttranslational modification

For a molecule to successfully act as a molecular switch, it must be able to relay—localize—information from the extracellular matrix into the cytoplasm and often to the nucleus. For Ras, localization to the cytoplasmic leaflet of a cellular membrane is essential for activation of the downstream effectors. [56] However, without any lipid attachments, Ras molecules are hydrophilic and cytosolic in nature. So, for Ras molecules to succeed in membrane localization, they must first be posttranslationally modified at the C-terminus. [57] Following C-terminus modifications, Ras are inserted into the hydrophobic tails of the phospholipid bilayer. [58]

The C-terminus of Ras is often described as the CAAX box, which consists of a cysteine and two aliphatic residues followed by any amino acid, respectively. Creating mature Ras molecules requires a four-step series that modifies each of the amino acids in the CAAX box. These steps are: 1) farnesylation, 2) proteolysis, 3) methylation, and 4) palmitoylation. First, a farnesyl is added to the cysteine by a farnesyl transferase which located in the cytoplasm. [59] This leads to cleaving or proteolysis of the –AAX by Ras Converting Enzyme 1

(RCE 1). Next the newly exposed C-terminus is methylated by Isoprenylcysteine Carboxyl Methyl Transferase (Icmt). [60, 61] HRas, NRas, and KRas 4A are further modified by a palmitoyl transferase that attaches a palmitate to the two cysteines just upstream of the CAAX box. [62-67] These modifications now create a hydrophobic C-terminus and, ultimately, permit membrane binding of Ras upon activation of the signal transduction cascade. [56] **Figure 3** shows the four steps of Ras posttranslational modification.

KRas 4B, the most abundant form of KRas, lacks these palmitoylation sites. Instead the farnesyl is sufficient enough to localize KRas 4B to the membrane and then the positive charges on the polybasic residues (-KKKKKKSKTK) help insert KRas 4B into the cytoplasmic face of the membrane. [2] This unique polybasic region allows KRas 4B to behave differently than other Ras isoforms. KRas localizes to the membrane in an unknown mechanism whereas other Ras are traditionally thought to localize to the plasma membrane via vesicular trafficking. [68-70]

Ras signal transduction

Ras, like many other small GTPase family members, is activated by its GTP and GDP bound state. [71] GTP binding increases Ras' affinity for the down stream effectors through conformational changes in the switch 1 and switch 2 regions. (**Figure 1**) GTP binding is a very transient phenomenon that is facilitated by Guanine Nucleotide Exchange Factor (GEF). A GEF releases the GDP already bound to Ras which allows Ras to exchange GDP for a GTP. [72] The reverse is facilitated by GTPase Activating Protein (GAP), which acts by releasing the terminal phosphate group from the GTP and returns the Ras once again to its GDP bound form. [10] Amino acid changes in the switch regions that hinder this exchange often result in dominant negative and constitutively active mutants. Also, the ability of Ras to

behave as a GTPase is the primary difference between Ras and its oncogenic counterparts—activity decreases up to 300 fold in transformed cells. [4]

Because Ras activation results in various cellular responses, Ras signal transduction is a complicated process that requires specificity and precision. [73] Of all Ras signal transduction cascades, perhaps the best studied is the Mitogen Activating Protein Kinase (MAPK) cascade. In this cascade, an extracellular growth factor, such as Epidermal Growth Factors (EGF), binds to its receptor (EGFR), which causes dimerization and phosphorylation. [74] In response to the phosphorylation, Growth Factor Receptor Binding Protein (Grb), an adaptor protein, binds to Son of Sevenless (SOS), becomes a GEF/adaptor complex, and localizes to the plasma membrane. [75] Once at the plasma membrane, this GEF/adaptor complex activates Ras which, in turn, recruits and activates the serine/threonine kinase named RAF. [76] RAF is then phosphorylated and activates MAPK/ERK Kinase (MEK), then MEK activates Extracellular signal-Regulated Kinase (ERK). After phosphorylation, ERK is dimerized and that complex will move to the nucleus. Once in the nucleus, ERK activates transcription factors. [77] (**Figure 4**)

Ras mediated signal transduction from endomembranes

Traditionally, the final destination for activated Ras is thought to be the plasma membrane. [78] Any other localization on endomembranes was considered a temporary occurrence where pools of proteins had not yet been trafficked to the plasma membrane. [36] Although the plasma membrane may not be the only membrane platform, the plasma membrane is still the most prominent place where Ras signal transduction occurs. In fact, up to now, conventional thinking has been that Ras C-terminus mutants that improperly direct Ras to endomembranes (e.g., Golgi and ER) are less likely to be oncogenic. [79, 80]

However, recent studies have also shown that Ras can localize to other membranous organelles such as Golgi apparatus, endoplasmic reticulum (ER), and even mitochondria. [31] A study by Chiu et al. shows that Ras can be activated from both Golgi and the plasma membrane when stimulated. [52] Interestingly, while signaling was rapid and transient at the plasma membrane, signaling at the Golgi was slower and long lasting. [81]

In addition to the Golgi, the ER can also serve as a signaling platform for Ras. [82, 83] When Ras is mutated such that its farnesylation sites are retained but not its palmitoylation sites, the vast majority of the Ras protein localizes to the ER and cytosol. When these ER-bound proteins are expressed in conjunction with the Green Fluorescent Protein-Ras Binding Domain of RAF (GFP-RBD) and stimulated with growth factors, rapid accumulation of Ras on the ER has been observed. [84] This kind of Ras colocalization experiment with GFP-RBD demonstrates that not only have the ER-redirected Ras molecules retained their GTPase activity but they also maintain their signal transduction capabilities.

There is strong evidence that the plasma membrane is not the only platform for Ras-mediated signal transduction cascade. Ras farnesyl inhibitors that are developed to prevent only Ras plasma membrane localization might be inadequate in terms of Ras localization at other endomembrane structures.

Vesicular endocytosis

Once the Ras signaling cascade is transmitted at the plasma membrane, Ras is endocytosed then it is either recycled back out to the plasma membrane or degraded. [85, 86] This vesicular trafficking process provides a method for desensitization and down regulation which is essential for cell survival. [87-89] There are two basic modes of endocytosis—

phagocytosis and pinocytosis. Endocytic vesicles converge at the lysosome where they can be degraded or at the endosomal recycling center. [90] (**Figure 5**)

PHAGOCYTOSIS

Phagocytosis generally occurs in specialized cells that interact with large cellular debris and/or pathogens. Phagocytosis might be also responsible for how eukaryotic cells first obtained mitochondria and chloroplasts.

PINOCYTOSIS

Pinocytosis is responsible for the cell's fluid and small nutrient uptake. Pinocytosis can be further divided into four categories: 1) clathrin-mediated, dynamin-dependent, 2) caveolin-mediated, dynamin-dependent, 3) clathrin/caveolin-independent but dynamin-dependent, and 4) clathrin-, caveolin-, and dynamin-independent.

Figure 6 shows phagocytosis and the four categories of smaller pinocytosis vesicles.

1. Clathrin-mediated, dynamin-dependent endocytosis. Among the four categories of pinocytosis, perhaps the clathrin-mediated endocytosis (CME) is the best understood.

Endocytosis is initiated when the Adaptor Protein complex2 (AP2) is recruited to the plasma membrane after the ligand binds to its receptor. This allows the clathrin triskelia to form a lattice around the AP2/ligand/receptor complex. [91-93] The clathrin lattices create plasma membrane curvatures that eventually become external phases of the vesicles. In order for these budding vesicles to completely separate from the plasma membrane, they require dynamin, yet another GTPase,. Dynamin forms a *necklace* around the neck of the budding vesicle and pinches off the nascent vesicle from the plasma membrane. [92, 93] Both Epidermal Growth Factor Receptor (EGFR) and transferrin are thought to be endocytosed through this pathway.

2. Caveolin-mediated, dynamin-dependent. The caveolin-mediated, dynamin-dependent category of pinocytosis results in caveolae vesicles. These coated, endocytic vesicles are lined with caveolin, which dimerizes and binds to cholesterol, and inserts the caveolae into the cytoplasmic side of the plasma membrane. Caveolae are tightly regulated by endocytosis pathways.

3. Clathrin/caveolin-independent but dynamin dependent. The third type of pinocytosis vesicles are clathrin and caveolin independent, but require dynamin, a small GTPase. Unlike clathrin, dynamin does not coat the endocytic vesicles but activates a budding off mechanism that releases vesicles from the plasma membrane. A dynamin-dominant-negative mutant that cannot exchange its GDP for GTP inhibits vesicular endocytosis.

4. Clathrin/caveolin/dynamin-independent. The final pinocytosis category is independent of dynamin, clathrin, and caveolin. [94] This pathway is thought to be mediated via ARF (ADP Ribosylation Factor). [95] ARF is a small GTPase that is no stranger to vesicular trafficking. [96] ARF contains six family members—ARF1, ARF2, ARF3, ARF4, ARF5, and ARF6. Of the six, ARF1 and ARF6 have been well studied. ARF1 is a Golgi-localized protein that directly interacts with COP I in Ras exocytosis. On the other hand, ARF6 is thought to regulate endocytosis and trafficking to the ERC which is embedded within the Golgi complex. [97] (**Figure 6**)

ENDOSOMAL RECYCLING CENTER

Regardless of the vesicular coating or lack thereof, once separated from the plasma membrane, vesicles travel toward the cytosol and cluster together to form the Early Endosomal Compartment (EEC). [98] From the EEC, cargo-containing vesicles can either join the late-endosome, or eventually localize to the lysosome where the proteins can be

degraded or the cargo vesicles can be localized to the ERC where cargos such as EGFR are redirected toward the plasma membrane. [85] Even though Ras signaling and trafficking out to the plasma membrane are well known, the fate of Ras after signaling has been elusive. In addition, because vesicular membranes are derived from the plasma membrane, it is reasonable to speculate that Ras can continue to signal while bound to a vesicular membrane as long as all of its signaling partners are present. [99] Furthermore, even if Ras effectors are not present in the exact same vesicle as Ras, and because several vesicles merge to form early endosomes (EE), Ras can encounter its downstream effectors and use EE as a signaling platform. Finally, because some proteins can be localized to the ERC [52], HRas-mediated signal transduction can also occur there as long as Ras and its effectors are both located in the ERC. **(Figure 5)**

Vesicular exocytosis

Generally, exocytosis describes movement of vesicles from inside the cell toward the plasma membrane and out into the extracellular matrix. Currently we recognize two kinds of protein trafficking, ER to Golgi and Golgi to ER.

CARGO TRANSPORT FROM ER TO GOLGI

Trafficking between ER and Golgi has been well documented. [38, 39, 100, 101]

Trafficking between these two membranes is interesting because both anterograde trafficking (ER to Golgi) and retrograde trafficking (Golgi to ER) occur simultaneously. [102, 103] For example, ER to Golgi anterograde trafficking requires small GTPases such as Sec-related and Ras-related protein 1 (Sar1). [45] To initiate anterograde trafficking between ER and Golgi, GTP binds to Sar1 and allows the COP II complex of proteins to accumulate. [44] These aggregations of COP II proteins complexes now mark the ER exit sites. [104] It is here, in these ER exit sites, that the cargo-containing vesicles will begin to form. The GTP binding of

Sar1 serves as an initiation signal for other GTPases such as ARF 1 [105, 106], which is required for vesicle maturation and, eventually, their release from ER. [107-110] Once formed, the cargo-containing vesicles are exported to the vesiculo-tubular intermediate compartment called Endoplasmic Reticulum Intermediate Compartment (ERGIC) before arriving at the Golgi. [107] From Golgi, the vesicles will continue to move toward the plasma membrane by using microtubules. [111, 112]

CARGO TRANSPORT FROM GOLGI TO ER

Golgi is a dynamic tubular structure that is used for cargo sorting and trafficking. One of the unique attributes of Golgi is its ability to distinguish between outward bound vesicles headed toward the plasma membrane and other vesicles that are headed toward other endomembranes, including ER. [113] Golgi is primarily formed by accumulating vesicles from the ER. [114-116] As with the ER, the Golgi apparatus has its own set of resident proteins (e.g., giantin), but they are not tightly bound to the membrane. [114] Golgi residents can often cycle back and forth between ER and Golgi. Thus, Golgi's structural integrity and its ability to maintain a steady pool of resident proteins are highly dependent on the continuous fusion of ER derived vesicles to the existing Golgi. [42-45, 48] In some cases, if Golgi and its residents are not replenished by constant forward and backward trafficking, it will disassemble. The retrograde trafficking from Golgi back to ER is facilitated by the COP I machinery. [117]

One of the commonly used pharmacological reagents in protein trafficking studies which targets this constant exchange of vesicles between ER and Golgi, is Brefeldin A (BFA), a fungal reagent that the COP I coated vesicles are especially sensitive to. [118, 119] More specifically, BFA prevents GTP binding of ARF 1. When ARF 1 activation is inhibited, COP

I machinery is prevented from forming and designating ER export sites. [120] Ultimately, exposure to BFA will arrest vesicular budding from ER. Even though this does not prevent Golgi-to-ER anterograde trafficking. Upon prolonged BFA treatment, the Golgi membranes retreat quickly back into the ER and are not replenished. So, in time, the Golgi completely collapses. [119, 121]

Microtubules and intracellular trafficking

Microtubules are filamentous structures that are part of cytoskeletons and are important for maintaining cell structure, polarity, motility, and division. [112, 122] Microtubules are polymers made of heterodimer units that consist of α and β subunits. Although both subunits bind to GTP, only the β subunit is able to exchange GTP for GDP.

Microtubular polarity allows cargo to travel in only one direction. [123] The growing end of the microtubule, where elongation takes place, is called the plus end, and the shrinking end of the microtubule is called the minus end. Microtubules are regulated by the members of the GTPase family like Rho and Ran. Like Ras, Rho and Ran are also activated by GTP binding and inactivated by GDP binding. Expression of the dominant negative forms of Rho or Ran proteins—when the proteins cannot release the GDP—prevents microtubule polymerization. This effect can also be created by using pharmacological reagents like nocodazole. [124]

Powered by GTP, microtubules constantly cycle between elongation and shrinkage. [124] This cycling makes microtubules very unstable structures, but cycling also allows new tracks to be laid quickly so they can help healthy cells move and traffic vesicles. [125]

Concluding Remarks

Ras signals determine whether cell proliferation is regulated or is not regulated, in which case, cells become oncogenic, so study into Ras signaling offers insights into tumorigenesis, the focus of the three papers that make up the core of this dissertation.

The studies discussed here present evidence that suggests that Ras signals from multiple locations at multiple times and with multiple signaling partners. This adds more complexity to what was already a complex system, but because Ras can signal from multiple locations, understanding Ras signal transduction in relation to its trafficking pattern moves us closer to understanding oncogenesis at the cellular and molecular levels.

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Figure Legends

Figure 1. Ras exocytosis

A newly made Ras protein is located in the cytosol. Ras posttranslational modification begins with addition of a farnesyl group by the farnesyl transferase. This lipid modification kinetically traps HRas on the cytosolic surface of the ER membrane. Here the CAAX box is cleaved and two palmitates are added, so HRas posttranslational modification is complete. A mature HRas can move out to the plasma membrane by a vesicular trafficking mechanism, which includes passage through ERGIC and Golgi. Ras can also traffic to the plasma membrane directly from ER in a non-vesicular trafficking mechanism. KRas, which only contains a farnesyl group, localizes to the plasma membrane from ER in an unknown trafficking mechanism.

Figure 2 Ras domains

Structurally Ras proteins are divided into conserved domains and hypervariable regions. The conserved domain begins at the N-terminus and extends to residue 165. This domain is well conserved all Ras isoforms. Within the conserved domain there are two switch regions (switch 1 and switch 2) where GTP/GDP exchange occurs. Mutations in the switch regions can result in constitutively active or dominant negative mutants. The hypervariable region begins at the residue 166 and ends at the C-terminus. This region is posttranslationally modified with lipid groups. KRas 4 B acquires a farnesyl group at the CAAX box, whereas KRas A and NRas acquire an additional palmitate just upstream. HRas acquires two palmitates in addition to the farnesyl.

Figure 3 HRas C-terminus modifications

A newly synthesized HRas must be posttranslationally modified at the C-terminus before acting as a molecular switch. HRas posttranslational modification begins with the addition of a farnesyl group at the C of the CAAX box by the farnesyl transferase. Then the —AAX of the CAAX box is cleaved by the Ras Converting Enzyme 1 (RCE 1), and the newly exposed C-terminus is methylated by the Isoprenyl cysteine Carboxy Methyl Transferase (Icmt). Finally, two additional palmitates are added to the upstream cysteines by palmitoyl transferase and complete HRas C-terminus modifications.

Figure 4 Ras-mediated MAPK signal transduction pathway

The best studied Ras-mediated signal transduction is the Mitogen Activating Protein Kinase Pathway (MAPK). To begin the signal transduction cascade by activating Ras, a GEF exchanges a GDP for a GTP, then the Ras downstream effectors RAF, MEK and ERK activated by addition of phosphate groups. Activation of this signal cascade leads to cell proliferation, differentiation, and apoptosis. After initiating the MAPK signal transduction pathway, Ras becomes inactive by exchanging its GTP for a GDP. This exchange is facilitated by GAP enzymes.

Figure 5 Endocytic vesicle trafficking

There are two major endocytic vesicle trafficking pathways—clathrin and non-clathrin.

In the clathrin trafficking pathway, clathrin coated vesicles are released from the plasma membrane and become an Early Endosome (EE). An EE can continue its trafficking into the cytosol and become a late endosome or it can merge with other vesicles and form a merged endosome. A merged endosome can either traffic to the Endosomal Recycling Center (ERC) embedded in the Golgi or the merged endosome

can traffic to the lysosomes along with late endosomes where the endosomal contents are degraded.

Figure 6 Types of endocytic vesicles

Endocytic describes a process in which cells take in materials from the extracellular matrix into the cytosol along with a small portion of their plasma membrane.

Phagocytosis and pinocytosis are the two major types of endocytosis. In phagocytosis, structures like whole cells and cell debris are engulfed. Whereas in pinocytosis, smaller molecules are engulfed into the cytosol. There are four major types of pinocytosis: clathrin mediated, dynamin-dependent; caveolin mediated, dynamin-dependent; clathrin and caveolin independent, dynamin-dependent; and clathrin, caveolin, and dynamin-independent. Clathrin and caveolin are types of vesicle coats and dynamin is a small GTPase that releases the vesicles from the plasma membrane.

Figures

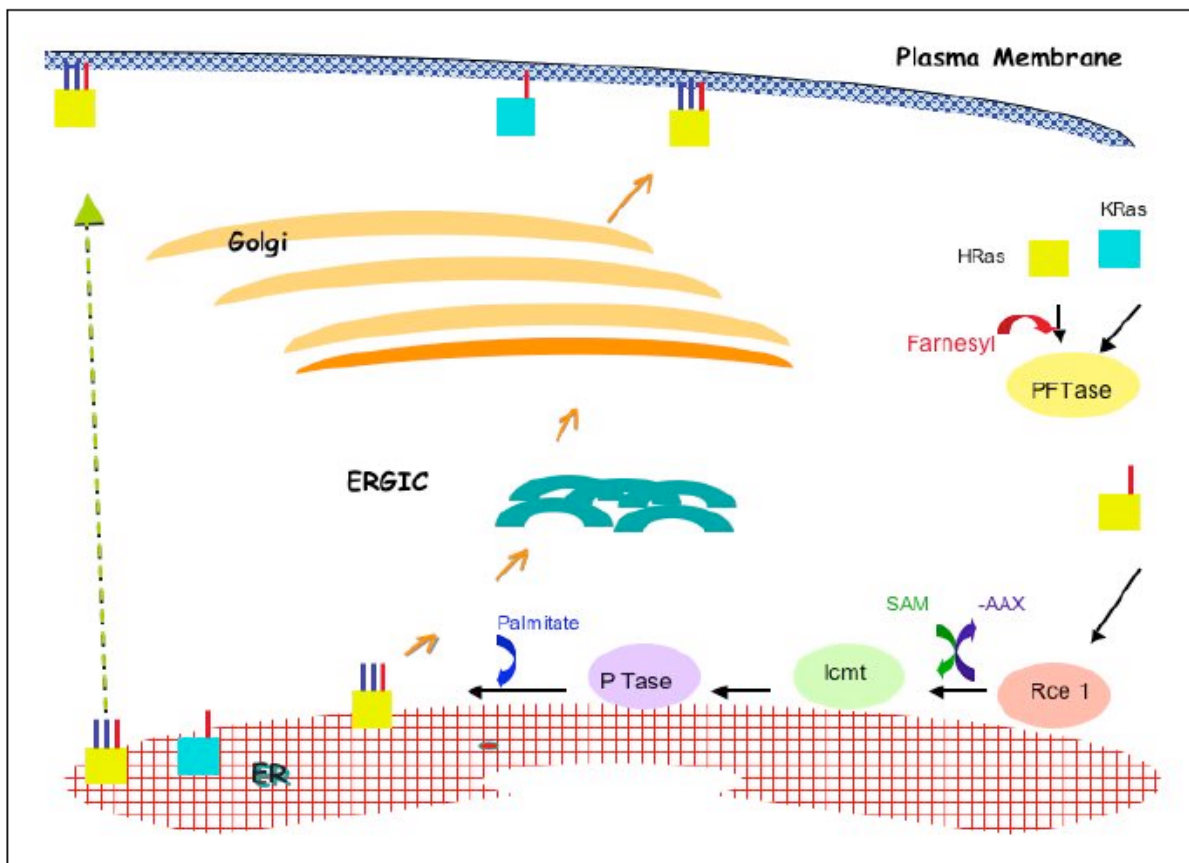


Figure 1 Ras exocytosis

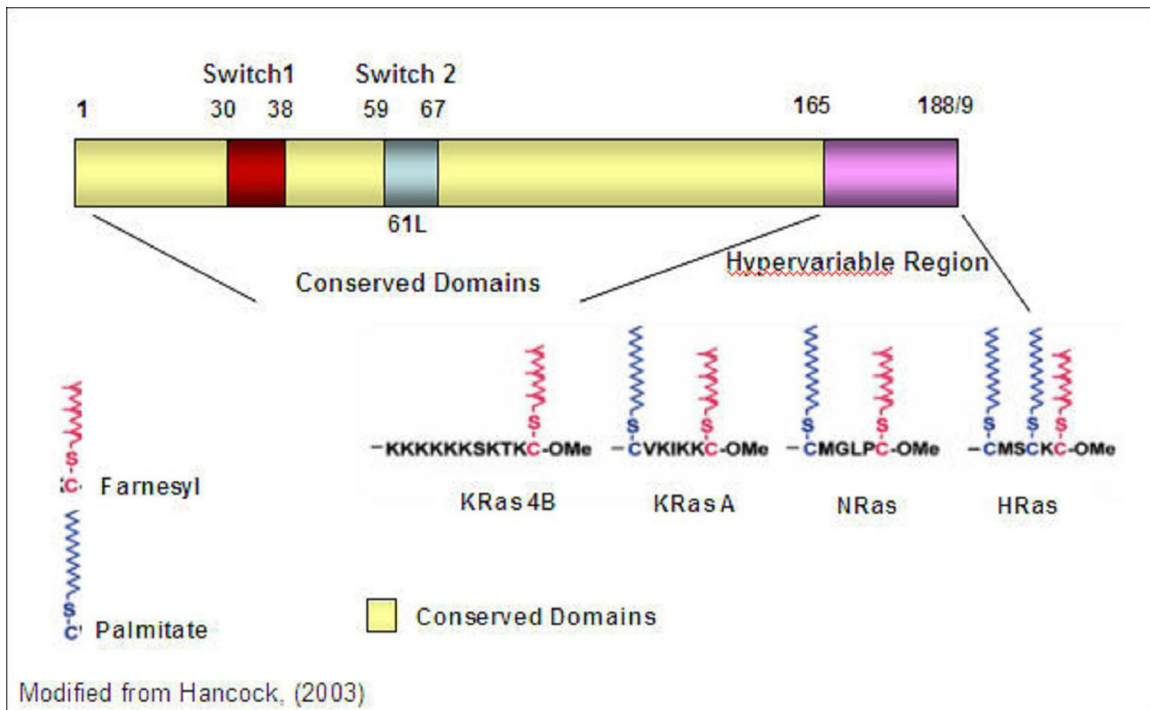


Figure 2 Ras domains

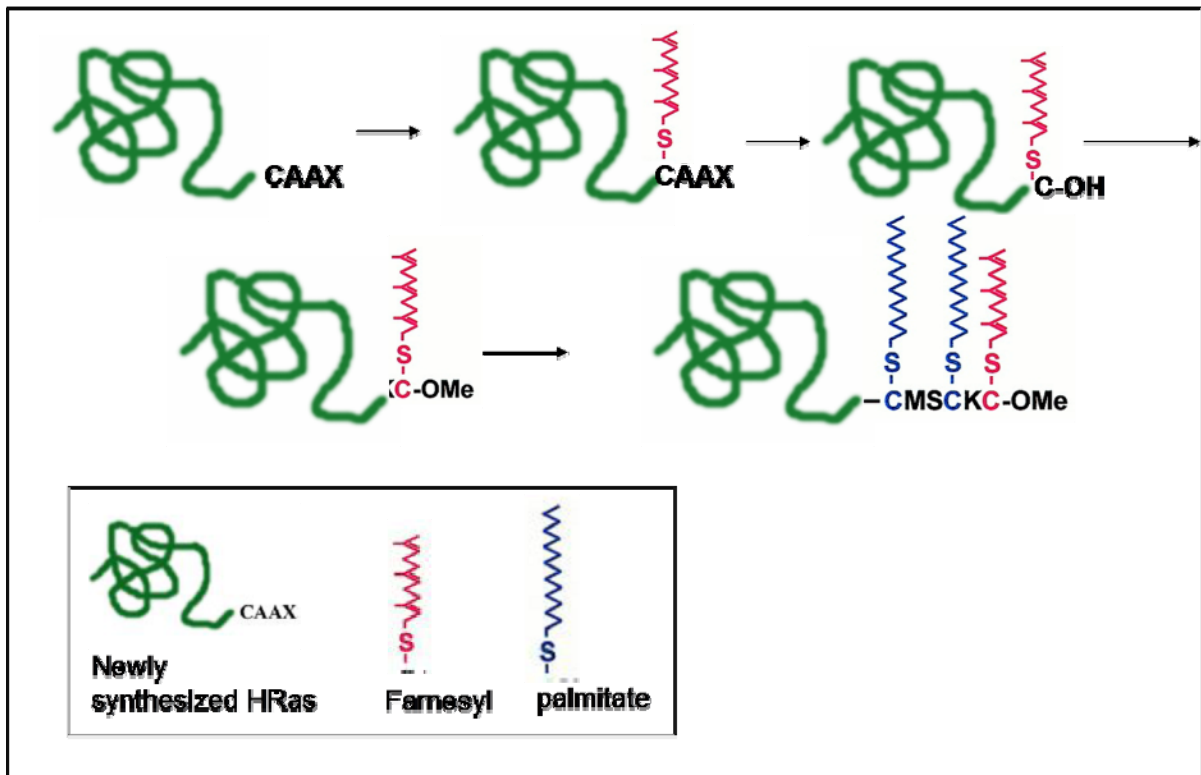


Figure 3 HRas C-terminus modifications

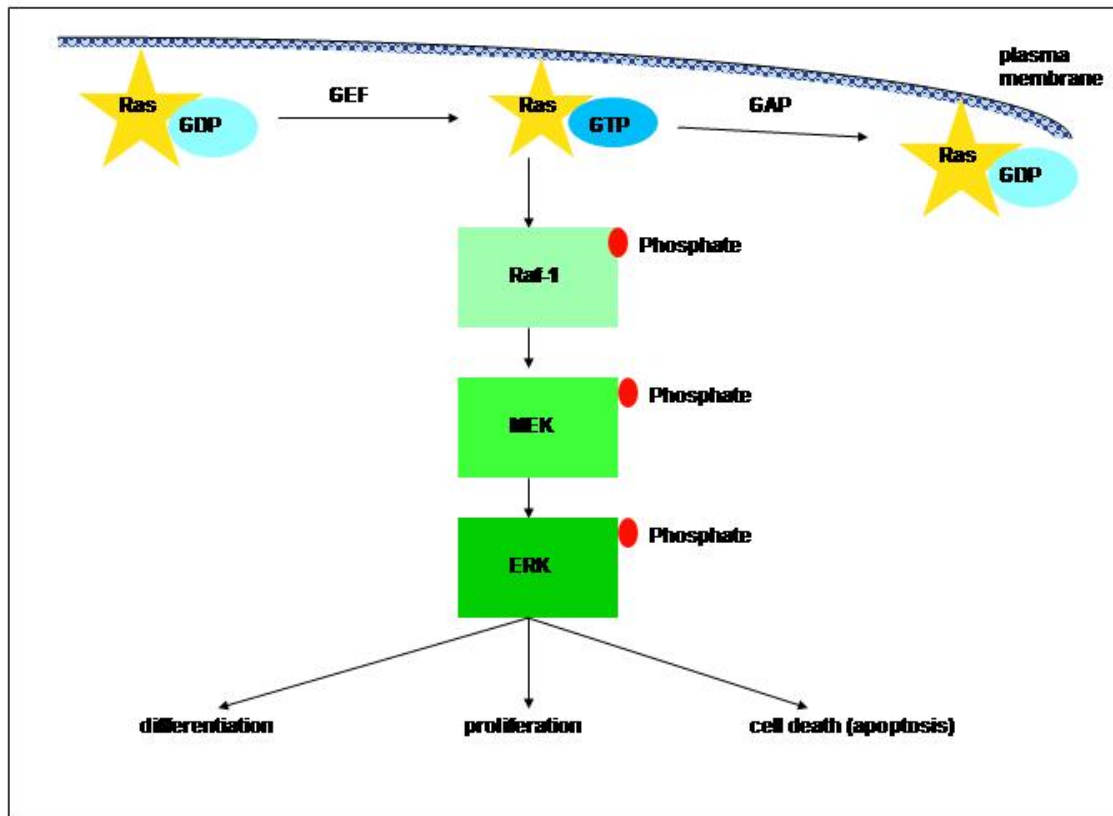


Figure 4 Ras-mediated MAPK signal transduction pathway

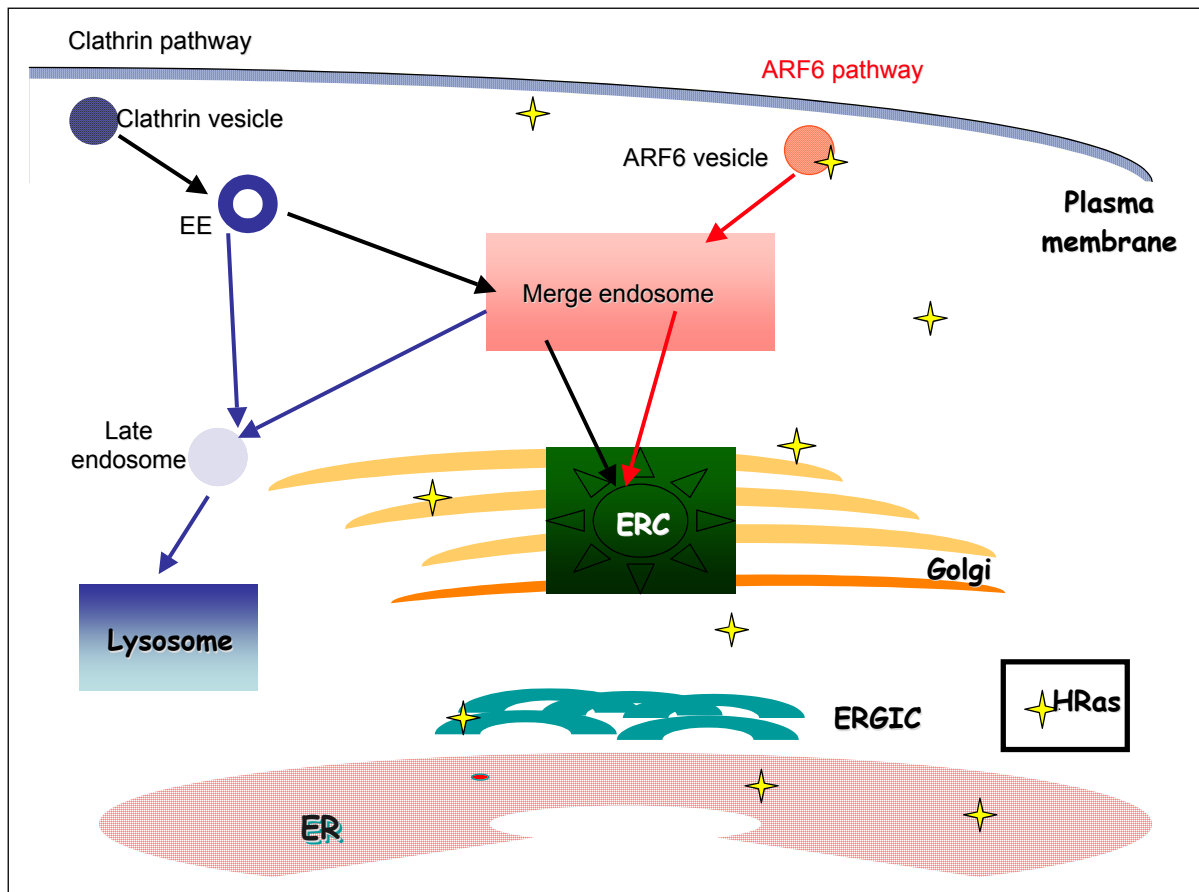


Figure 5 Endocytic vesicle trafficking

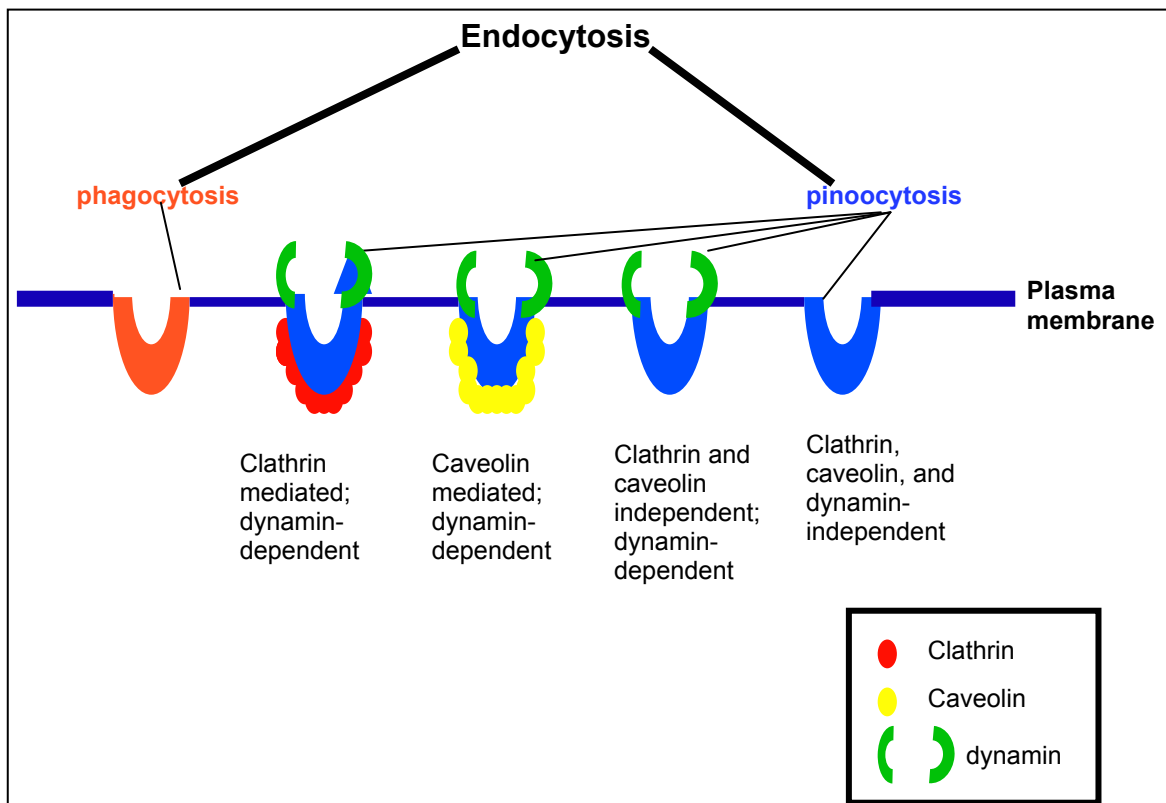


Figure 6 Types of endocytic vesicles

H-Ras does not need COP I- or COP II-dependent vesicular transport to reach the plasma membrane

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Running Title: COP I- and COP II-independent H-Ras trafficking

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Abstract

Although vesicular transport of the H-Ras protein from the Golgi to the plasma membrane is well-known, additional trafficking steps, both to, and from, the plasma membrane, have also been described. Notably, both vesicular and non-vesicular transport mechanisms have been proposed. The initial trafficking of H-Ras to the plasma membrane was therefore examined in more detail. In untreated cells, H-Ras appeared at the plasma membrane more rapidly than a protein carried by the conventional exocytic pathway, and no H-Ras was visible on Golgi membranes in >80% of the cells. H-Ras was still able to reach the plasma membrane when COP II-directed transport was disrupted by two different mutant forms of Sar1, when COP I-mediated vesicular traffic from the ER to Golgi was inhibited with Brefeldin A, or when microtubules were disrupted by nocodazole. Although some H-Ras was present in the secretory pathway, protein that reached the membranes of the ER-Golgi intermediate compartment was unable to move further in the presence of nocodazole. These results identify an alternative mechanism for H-Ras trafficking that circumvents conventional COP I-, COP II-, and microtubule-dependent vesicular transport. Thus, H-Ras has two

simultaneous but distinct means of transport, and need not depend on vesicular trafficking for its delivery to the plasma membranes.

Introduction

The broad outlines of the biosynthetic transport of Ras proteins to the plasma membrane are known [1-3]. Mammalian H-Ras, N-Ras, and K-Ras and also yeast Ras proteins are initially cytosolic, become modified with a farnesyl isoprenoid, and are then escorted, perhaps by the farnesyl transferase itself [4,5], to the cytosolic face of the widely dispersed membranes of the endoplasmic reticulum. There, farnesylated Ras proteins are further modified by an ER-resident prenyl-dependent protease (Rce1), and by a second ER-resident methyl transferase enzyme (Icmt). [6,7]. At this point the K-Ras4B protein is released from the surface of the ER and rapidly appears at the plasma membrane. [3,8,9]. No internal membrane or vesicular intermediates have been observed for this step, and the transport mechanism is currently unknown. [10]. In neural cells, calcium signals cause calmodulin to bind to plasma membrane-bound K-Ras4B and trigger its internalization through a protein-mediated, cytoplasmic shuttle mechanism. [11]. Thus K-Ras4B, at least in neurons, appears to use non-vesicular means for both initial outward and signal-dependent, inward trafficking.

For H-Ras, N-Ras and yeast Ras2, a second lipid modification can occur, in which a palmitoyl lipid is attached to additional cysteines near the farnesylated C-terminus. A two-subunit protein acyl transferase enzyme that attaches palmitates to H-Ras and N-Ras in mammalian cells has only recently been identified, and may be located on ER and Golgi membranes. [12-14]. In yeast, a similar two-subunit enzyme (Erf2/Erf4) that modifies yeast Ras2 has been found on ER membranes. [15]. Another mammalian acyl transferase, Huntington interacting protein-14 (HIP-14), is distributed on ER/Golgi membranes and has

been reported to enhance vesicular trafficking of multiple acylated proteins. [16,17].

Additional palmitoyl transferase enzymes are likely to be located in the plasma membrane, because mutant yeast and mammalian Ras proteins that reach the plasma membrane without associating with ER or Golgi membranes can be palmitoylated. [18,19].

The movement of H-Ras from the cytosolic surface of the vast ER membrane network is the first step in its journey to the plasma membrane. Two recent reports have laid the framework of this path. These studies propose that non-palmitoylated (but farnesylated) H-Ras distributes rapidly between cytosolic and transient, non-specific membrane-bound phases. Wherever H-Ras encounters an acyl transferase and is palmitoylated it becomes "kinetically trapped" on the membranes [20], and as a result, can then be retained on vesicles and moved onward via traditional vesicular means. [21,22]. Proteins that control vesicular trafficking at the sites where acyl transferases are found thus become relevant for H-Ras transport. However, the model currently does not address whether a farnesylated H-Ras will need a partner protein to mask its lipid group during its cytosolic phase. These possibilities give importance to understanding the steps that H-Ras takes as it makes its way from the ER to the plasma membrane.

A great deal is known about vesicular trafficking from the ER to the Golgi. Formation of vesicles on the ER begins with assembly of a COP II complex of proteins around the site, termed an ER exit site (ERES), at which the newly cargo-loaded vesicle will form. [23-25]. The protein that initiates this event is the GTPase, Sar1. GTP-binding mutants of Sar1 disrupt this cycle, and inhibit COP II-mediated vesicle transport from ER to Golgi. [26]. In mammalian cells, the COP II vesicles that are released from the widely dispersed ERES then seem to merge into a perinuclear vesiculo-tubular center sometimes termed the ER-Golgi-

intermediate compartment (ERGIC). From here, some vesicles move onward to the Golgi, while others return to the ER. [27].

COP I-coated vesicles mediate anterograde transport from the ERGIC to Golgi, and retrograde traffic from Golgi membranes to the ER. [28,29]. The fungal product Brefeldin A (BFA) prevents the COP I complex from binding to membranes. This causes collapse of the Golgi stacks, as the membranes and proteins of the Golgi rapidly drain backwards into the ER, and also impairs anterograde trafficking beyond the ERGIC. [30] Thus, BFA inhibits vesicular transport at a separate point and by a mechanism distinct from that of Sar1 mutants.

BFA has been reported to partially inhibit transport of a chimeric GFP protein with a lipidated C-terminus derived from H-Ras. [3]. However, another study reported continued transport of both H-Ras and another GTPase, TC10, to the plasma membrane in BFA-treated cells. [31]. Thus, it is unresolved to what extent BFA-sensitive, conventional vesicular trafficking contributes to H-Ras transport. The yeast Ras2 protein (which is modified by both farnesyl isoprenoid and palmitoyl lipids) has been reported to use a non-conventional pathway for trafficking. [19].

These precedents prompted us to examine the mechanisms by which mammalian H-Ras accesses the plasma membrane, with particular attention to the initial steps at its release from the ER. The results indicate that two distinct routes for H-Ras transport operate simultaneously, and that a non-conventional, COP I- and COP II-independent mechanism moves the bulk of H-Ras to the plasma membrane. Unexpectedly, when traditional vesicular trafficking is blocked, H-Ras that has entered that pathway appears unable to switch to the non-conventional pathway. This suggests that the choice of pathway, and mechanism of transport, is determined prior to ER disengagement.

Materials and Methods

Mutants, Plasmids and Antibodies

- H-Ras^{wt}, H-Ras^{Q61L}, and GFP-H-Ras were expressed from pcDNA3 (Invitrogen). The cDNAs for YFP-GT46 and the monomeric GFP (mGFP) [32] were gifts from Anne Kenworthy (Vanderbilt), with permission of Roger Tsien (San Diego). The cDNAs for hemagglutinin (HA)-tagged Sar1^{T39N}-HA and Sar1^{H79G}-HA were gifts from Dr. Jennifer Lippincott-Schwartz (NIH). Anti-Ras rat monoclonal antibody 238 (Santa Cruz), anti-HA mouse monoclonal antibody (Covance), anti-giantin rabbit polyclonal antibody (Covance), anti-ERGIC-53 mouse monoclonal antibody (Alexis), and Alexa-Fluor conjugated secondary antibodies (Molecular Probe) were used for immuno-fluorescence. The anti-Ras rat monoclonal antibody was detected with a rat-specific secondary antibody.

Cell Culture and transfection

NIH 3T3 cells and COS-7 cells were cultured at 10% CO₂ in Dulbecco's Modified Eagle's Medium (GIBCO-BRL) supplemented with 10% calf serum (Hyclone Laboratories). Twenty-four hours before DNA transfection, cells were plated on pre-washed glass cover slips (Corning) in 12 well tissue culture plates at a density about 10⁵-10⁶ cells per well. Transfection was performed using Effectine reagent (GIBCO-BRL) as described by the manufacturer. For sequential DNA transfection, only Sar1 DNA was used for the first transfection, and after 18 hours a second round of transfection, with DNA for H-Ras, was performed. For Sar1 co-transfection, a DNA ratio of 2:1 (Sar1: other DNAs; w/w) was used. For all other co-transfections a 1:1 DNA ratio (w/w) was used.

Counting cells

NIH 3T3 cells were co-transfected with cDNAs for YFP-GT46 and H-Ras, as indicated. After 18 hours, cells were fixed and viewed by epifluorescence (for GFP constructs) or

immuno-stained with antibody for H-Ras or giantin. One hundred cells with YFP-GT46 (or giantin) visible in the perinuclear area were then counted, and the number of cells in which H-Ras was present only on plasma membrane or coincident with the perinuclear marker protein was noted. Two separate experiments were performed for each H-Ras form, and the data were analyzed using the GraphPad Prism software program.

Brefeldin A, nocodazole and cycloheximide treatment

At 6 hours after transfection, Brefeldin A (BFA, 5 $\mu\text{g/ml}$) was applied to the cells in regular medium. Cells were fixed and visualized by immuno-fluorescence at 30, 60 and 90 minutes after BFA treatment. Where indicated, cells were treated with cycloheximide (50 $\mu\text{g/ml}$) immediately after the transfection. After 6 hours the cycloheximide was washed out and fresh medium with either BFA or nocodazole (20 $\mu\text{g/ml}$) was added. After 1.5 hours, cells were fixed for immuno-fluorescence. For one experiment, cells were transfected and treated with cycloheximide for 6 hours, then were rinsed to wash out the cycloheximide, and nocodazole was applied for 4 hours. Then nocodazole was washed out, and cycloheximide was added to the medium again. After 2 more hours, cells were fixed and stained for immuno-fluorescence.

Immuno-fluorescence imaging

NIH 3T3 and COS-7 cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) at room temperature for 15 minutes. Cells were permeablized with 0.2% TritonX-100 on ice for 5 minutes and treated with 50mM NH_4Cl in PBS for 15 minutes at room temperature. Non-specific binding was blocked using 1% fat-free milk in PBS for 15 minutes, followed by primary antibody for 1 hour and secondary antibody for 1 hour at room temperature. Cover slips were mounted with VECTASHIELD and images were captured

with a Leica inverted fluorescence microscope (Model DIMER2) or Leica confocal TCS NT microscope system equipped with separate Argon-UV, Argon, and Krypton lasers and the appropriate filter sets for FITC, Texas Red and TRITC imaging. The contrast and signal strength of images were balanced and images were deconvolved and merged as indicated, using Improvision Open Lab and Adobe Photoshop software.

Movies

NIH 3T3 cells were cultured and transfected with GFP-H-Ras cDNA as described before. Cells were treated with cycloheximide (50 μ g/ml) immediately after the transfection. After 6 hours the cycloheximide was washed out and fresh medium with BFA (5 μ g/ml) and HEPES (20 μ g/ml, pH 7.4) was added. Cells were thereafter kept at 37°C in a warmed chamber. After 30 minutes of BFA treatment, images of a selected cell were taken at 90-second intervals for about 1 hour with a Leica inverted fluorescence microscope (Model DIRME2). Movies were created by Improvision Open Lab software.

Results

H-Ras proteins often show little accumulation in the Golgi

Although previous reports clearly documented the presence of H-Ras on the ER and Golgi, most of those studies used GFP-H-Ras fusion proteins to produce bright signals when localized to a small area. [1,3]. When a non-tagged version of H-Ras was imaged, using an H-Ras antibody, many transfected NIH 3T3 cells showed H-Ras protein only on the plasma membrane, with no protein detectable on perinuclear membranes. This visual impression was quantified, by determining the proportion of cells that showed H-Ras immunofluorescence on Golgi membranes. To verify the location of the Golgi apparatus, a co-expressed YFP-GT46 protein, which traffics through the conventional exocytic pathway [33], or giantin, a resident

cis-Golgi protein, were also imaged. At 18 hours after transfection, H-Ras was strongly visible on the plasma membrane in >95% of all transfected cells. YFP-GT46 was detectable at the plasma membrane, and also in an organized perinuclear area in >90% of the transfected cells (Table 1). One hundred cells that showed YFP-GT46 in the perinuclear area were then counted, and the presence and abundance of H-Ras on membranes that were coincident with the perinuclear YFP-GT46 was noted in each. The results verified the preliminary impression that H-Ras was frequently absent from internal membranes (Golgi or recycling center [34]), as ~85% of the transfected cells showed no H-Ras on organized internal membrane structures that were also marked by YFP-GT46. Roughly 10% of the cells showed some modest amount of internal H-Ras, while about 5% showed strong colocalization with perinuclear membranes containing YFP-GT46. The same experiment was performed using COS-7 cells, with similar results (Table 1). Thus, in both cell types, only a few of the transfected cells showed a strong H-Ras signal on internal membranes.

To see if a GTP-bound, oncogenic form of H-Ras behaved similarly, an activated version, H-Ras^{Q61L} was expressed. Once more, in 85-95% of the transfected cells, there was very little H-Ras^{Q61L} that colocalized with YFP-GT46 in the perinuclear area, although the plasma membrane was illuminated (Fig. 1A; Table 1). Thus, both the native form and an oncogenic form of H-Ras displayed limited accumulation on internal membranes.

To look more specifically at the Golgi, the experiment was repeated, using COS-7 cells and staining of the endogenous protein, giantin, to mark the cis-Golgi. Again, a large majority of the cells showed almost no H-Ras^{Q61L} on Golgi membranes (Fig. 1B; Table 1). A similar limitation in Golgi localization of H-Ras has been reported in CHO-K1 cells. [35].

In contrast, when GFP-H-Ras fusion proteins were examined, a larger portion (~70%) of transfected cells showed the GFP-H-Ras fusion proteins on Golgi membranes, and only ~30% of the cells lacked any accumulation of H-Ras in the Golgi area (Table 1). This significant amount of GFP-H-Ras on Golgi membranes agrees with previous work using GFP-H-Ras proteins. [1]. To learn if this apparent influence of GFP on H-Ras localization might arise because of the tendency for the original versions of GFP to dimerize, a monomeric form of GFP was attached to H-Ras^{Q61L} and the steady-state distribution of this mGFP-H-Ras^{Q61L} was examined. In roughly half of the cells, mGFP-H-Ras^{Q61L} was visible on perinuclear membranes, compared to ~65% for the original GFP version. Meanwhile, the portion of cells in which no perinuclear fluorescence of mGFP-H-Ras was visible increased moderately, from ~35% for the original GFP-tagged version, up to about 50% with monomeric GFP version (Table 1). However, the mGFP-H-Ras^{Q61L} was still clearly different in its distribution from that of the untagged H-Ras^{Q61L}, in which <5% of the cells showed perinuclear fluorescence and >90% did not. These results suggest that, although GFP-H-Ras (or mGFP-H-Ras) proteins may be useful for documenting the presence of H-Ras at internal locations, they may not fully replicate normal usage of the transport pathways.

Nascent H-Ras moves much faster than YFP-GT46 to the plasma membrane

These results suggested two possibilities: a) in 85% of the cells the amount of H-Ras on Golgi membranes was low because vesicular transport was particularly efficient; or b) a significant amount of H-Ras might be able to access the plasma membrane without utilizing the exocytic pathway through the Golgi.

To study trafficking efficiency, the initial movement of H-Ras was examined at early time points after the protein was synthesized. This avoided the complexity of possible

deacylation-based recycling of previously synthesized H-Ras between plasma and internal membranes. [21]. For this, cells were co-transfected with cDNAs for H-Ras^{Q61L} and YFP-GT46 and then were placed immediately in medium containing cycloheximide for 6 hours, to allow the cDNAs to be transcribed, and the RNA for H-Ras to accumulate, but to prevent translation. Cycloheximide was then removed to produce a synchronous wave of H-Ras protein synthesis and trafficking. At various time points after cycloheximide removal, cells were fixed and imaged. Using this approach, both H-Ras and YFP-GT46 had been translated and accumulated in amounts sufficient to be visible within 50 minutes of removal of cycloheximide. At that time, both proteins were present only on the ER (**Fig. 2, 50'**). Twenty minutes later (70 minutes after cycloheximide removal) a strong signal of YFP-GT46 was present on well-organized perinuclear membranes in ~90% of the transfected cells (**Fig. 2, GT46, 70'**). Importantly, no visible YFP-GT46 had yet reached the plasma membrane. In contrast, H-Ras was already detectable on the cell surface, well ahead of YFP-GT46 (**Fig. 2, H-Ras, 70'**). By 90 minutes after cycloheximide removal, YFP-GT46 was present in the Golgi and, in some cells, on vesicles that appeared to be exiting the Golgi, but which were not yet at the cell surface (**Fig. 2, GT46, 90'**). At this time point, H-Ras was now easily visible at the plasma membrane (**Fig. 2, H-Ras, 90'**), although no H-Ras-containing vesicles were detected and fewer than half of the cells showed any H-Ras in the perinuclear area. After another 20 minutes, (110 minutes after cycloheximide removal), exocytic vesicles containing YFP-GT46 could be seen in most transfected cells. In some cells, these GT46-containing vesicles had reached the cell surface, and the outline of these cells could now be barely seen (**Fig. 2, GT46, 110'**).

H-Ras was now present on plasma membrane and on both perinuclear membranes and individual “granules” inside the cell (**Fig. 2, H-Ras, 110'**). When the images were merged, many of the vesicles containing YFP-GT46 did not have H-Ras on them (**Fig. 2, merge, 110'**). This result suggested that much of the H-Ras was not using YFP-GT46-containing vesicles for transportation to the plasma membrane. By 140 minutes after cycloheximide removal, large amounts of YFP-GT46 had reached the plasma membrane and many YFP-GT46-containing vesicles enroute were obvious. Merged images of H-Ras and GT46 showed some vesicles that contained both H-Ras and YFP-GT46, although again, many contained only one of the proteins (**Fig. 2, merge, 140'**). The colocalization of YFP-GT46 and H-Ras agrees with previous reports that (some) H-Ras can traffic from the Golgi to the plasma membrane on vesicles. [1,21,22].

These results clearly showed that nascent H-Ras could reach the plasma membrane more rapidly than a protein carried on the traditional vesicular pathway, Furthermore, in many cells, there was no evidence that H-Ras transited internal membranes. This led us to test whether H-Ras could reach the plasma membrane if conventional vesicular transport was prevented.

Neither Brefeldin A nor dominant negative Sar1 prevent H-Ras movement to the plasma membrane

To test if H-Ras plasma membrane traffic required the presence or organization of the Golgi apparatus, brefeldin A was used to inhibit COP I-mediated vesicular transport and cause Golgi collapse. Cells were transfected with cDNAs for H-Ras^{Q61L} and YFP-GT46, and were immediately treated with cycloheximide. After allowing 6 hours for RNA synthesis, the cycloheximide was washed out to permit protein synthesis, but Brefeldin A (BFA) was also added. After an additional 90 minutes, the cells were fixed and imaged. As expected for a

transmembrane protein in the secretory pathway, YFP-GT46 was found in the ER after BFA treatment, and clearly outlined the ER membranes around the nucleus (**Fig. 3A**). No other internal membranes contained YFP-GT46, confirming that vesicular traffic had halted, and the Golgi apparatus had been absorbed into the ER. In contrast, H-Ras^{Q61L} continued to illuminate the plasma membrane in the presence of BFA (**Fig. 3A**). No organized, perinuclear structures that contained H-Ras^{Q61L} were visible, and the protein did not accumulate on the nuclear envelope. To determine if the activation state of the protein influenced whether H-Ras transport was BFA-insensitive, the experiments were repeated using the inactive, cellular form of H-Ras (H-Ras^{wt}). The results were similar to those with the activated form. BFA had no inhibitory effect on movement of H-Ras^{wt} to the plasma membrane (data not shown).

Additional trials with a 3 hr exposure to BFA were performed to examine if H-Ras might begin to accumulate on ER membranes if given more time. Even with this longer treatment, both H-Ras^{wt} and H-Ras^{Q61L} continued to move to the plasma membrane (data not shown). Also, BFA added to cells for 4 hours at 18 hours after transfection caused no accumulation of H-Ras on internal membranes (data not shown). Additional experiments with H-Ras^{Q61L} expressed in COS-7 cells showed that H-Ras^{Q61L} movement to the plasma membrane was not prevented by BFA in this other cell type (data not shown). These results indicated that neither GDP-bound nor GTP-bound forms of H-Ras required physically distinct Golgi membranes or COP I-coated vesicles in order to travel to the cell surface.

Although the results with BFA suggested that H-Ras might not require vesicular transport to reach the plasma membrane, these studies inhibited only the COP I side of this process. The necessity for H-Ras vesicular trafficking was tested in a second way, by preventing

assembly of the COP II complex, using mutants of the assembly regulator, Sar1. Cells were co-transfected with cDNAs for H-Ras^{Q61L} and a GDP-bound inhibitory form of Sar1 (Sar1^{T39N}-HA), and 24 hr later were fixed and imaged. The H-Ras^{Q61L} protein strongly outlined the plasma membrane of cells that over-expressed the Sar1 dominant-negative protein (**Fig. 3B**). There was no indication of accumulation of H-Ras^{Q61L} in the ER or around the nuclear envelope, nor any Golgi-like structures. This suggested indirectly that the Sar1^{T39N} had successfully ablated the Golgi. A second, longer experiment was also performed to give the Sar1^{T39N} more time to amass to levels that would certainly disrupt ER exit sites (ERES) before H-Ras was present. Even after 48 hr, in cells co-expressing Sar1^{T39N} and H-Ras^{Q61L}, the H-Ras^{Q61L} continued to mark the plasma membrane and showed no build-up in the ER (data not shown). In addition, a sequential transfection technique was developed to allow Sar1 more time to become functional before H-Ras was synthesized and began its trafficking. Cells were first transfected with cDNA for the Sar1^{T39N} and allowed to express this protein for 18 hr. The cells were then transfected a second time with cDNA for H-Ras^{Q61L} and after a further 24 hr, were fixed and imaged. All cells that expressed H-Ras^{Q61L} showed strong plasma membrane staining, including those that also co-expressed the previously synthesized Sar1^{T39N} (**Fig. 3B**). In cells that expressed both Sar1^{T39N} and H-Ras^{Q61L} proteins, there was no Golgi-like staining of H-Ras^{Q61L} on internal membranes. In cells that expressed only H-Ras^{Q61L} or that had only low expression of Sar1^{T39N}, there was again a mixture of cells displaying H-Ras on plasma membrane only, or having H-Ras on both plasma membrane and perinuclear membranes. H-Ras^{Q61L} did illuminate some scattered internal membranes that did not have the layered appearance of Golgi, and seemed to be fluid-filled pinosomes, with H-Ras-stained rims but dark interiors (**Fig. 3B**). Activation of H-

Ras is known to strongly stimulate fluid-phase endocytosis [36], so these structures may be the recently reported H-Ras^{Q61L}-containing endocytic vesicles [35] that had been internalized after reaching the plasma membrane.

To ensure that the Sar1^{T39N} protein was functioning correctly, a similar experiment was performed using the YFP-GT46 protein. The mutant Sar1 prevented YFP-GT46 from trafficking to the plasma membrane and caused it to accumulate on internal ER and nuclear envelope membranes (**Fig. 3B, lower row**). This indicated that the amount of Sar1^{T39N} that was expressed was sufficient to block transport of a protein in the traditional exocytic pathway. Equivalent results were obtained with Sar1^{T39N} and H-Ras^{Q61L} expression in COS-7 cells (data not shown), indicating that H-Ras trafficking is Sar1-independent in other cell types. To disrupt exit site assembly at a separate step, a different, GTP-bound Sar1 mutant, Sar1^{H79G}-HA, was employed. With either simultaneous or sequential transfection protocols, H-Ras^{Q61L} continued to reach the plasma membrane in cells that also expressed the Sar1^{H79G}-HA protein (data not shown). These results indicated that H-Ras did not need COP II vesicles in order to traffic to the plasma membrane.

Structures that carry H-Ras do not show directed movement to the plasma membrane in BFA-treated cells

The results to this point suggested that a great deal of H-Ras trafficking occurred via a pathway to the plasma membrane that was independent of the classical vesicular route through the Golgi. To examine this alternative pathway in more detail, the classical pathway was ablated with BFA, and a movie was recorded to study if any type of BFA-resistant vesicles carrying H-Ras protein could be seen moving to the plasma membrane. NIH 3T3 cells were transfected with GFP-H-Ras^{Q61L} cDNA and treated with cycloheximide immediately after the transfection. After 6 hours, cycloheximide was washed out and BFA

was added to the medium. After another 30 minutes to allow protein synthesis, the culture dish was moved to a chamber held at 37 degrees, and movement of the newly synthesized GFP-H-Ras^{Q61L} was recorded. As had been seen in the earlier experiments with fixed cells, at 40 minutes after the wash out of cycloheximide, detectable amounts of GFP-H-Ras^{Q61L} had already appeared in the cytosol and on ER membranes. The GFP-H-Ras^{Q61L} was also present on numerous, very small, granular structures within the cell. These granules were unlikely to be conventional vesicles as they did not arise from any organized perinuclear, Golgi-like center, and the previous experiments had shown that this time point was prior to the time at which (limited amounts of) H-Ras would be seen on conventional exocytic vesicles. The GFP-H-Ras^{Q61L} granules moved slowly, often inward (**Fig. 4, insets**), and had no specific direction towards the plasma membrane (**Fig. 4 and supplemental movie**). A few granules (two in the 8 cells that were imaged) did fuse with the plasma membrane and illuminate the membrane temporarily (data not shown). Notably, the GFP-tagged H-Ras protein could be seen on ruffles and plasma membrane of the BFA-treated cells. Thus, a GFP-tagged version of H-Ras could successfully access the plasma membrane during BFA exposure. This indicated that the GFP tag did not prevent this type of trafficking. As more GFP-H-Ras protein was synthesized after the removal of the cycloheximide, an increase in GFP-H-Ras fluorescence was noted in the perinuclear area. This area was not the Golgi, because it had been ablated by the BFA that was present. The identity of this region was examined below. The general absence of directed movement of the visible dots of GFP-H-Ras^{Q61L}, in addition to its COP I- and COP II- independence, suggested that the initial transport of GFP-H-Ras^{Q61L} to the plasma membrane that occurred in BFA-treated cells might be non-vesicular.

Nocodazole does not prevent H-Ras movement to the plasma membrane

To determine if transport of nascent H-Ras was also independent of microtubules that might support vesicular traffic, cells transfected with cDNA for H-Ras^{Q61L} were treated with cycloheximide for 6 hours, cycloheximide was removed and nocodazole was added for 90 minutes. With this treatment length, nocodazole causes fragmentation of the Golgi into multiple individual Golgi mini-stacks throughout the cell. These stacks were visualized with antibody to the endogenous Golgi protein, giantin, which verified that the nocodazole treatment had successfully dispersed the Golgi (**Fig. 5**). To determine how effectively this nocodazole treatment inhibited transport of a protein in the traditional exocytic pathway, GT46 was examined. After a 4-hour exposure to nocodazole, GT46 was found, exclusively, on internal membranes (**Fig. 5**); no GT46 was visible on the plasma membrane. This indicated that this protocol for nocodazole treatment efficiently inhibited microtubule-dependent vesicular transport during the experiment. Importantly, in cells in which microtubules had been depolymerized, H-Ras^{Q61L} was still able to reach the plasma membrane (**Fig. 5**). This indicated that a microtubule-independent mechanism could move H-Ras to the plasma membrane. These results complement previous work [22] that had shown that H-Ras that was already at the plasma membrane could reach the Golgi, when microtubules were depolymerized by acute application of nocodazole.

Notably, in these nocodazole-treated cells, there was a prominent amount of H-Ras that was located on dispersed internal structures. These HRas-containing structures were often alongside of, but did not actually colocalize with, the giantin-marked Golgi mini-stacks (**Fig. 5, merge**). This revealed that, although a significant amount of H-Ras reached the plasma membrane, nocodazole treatment caused some H-Ras to be retained internally, on structures

that were distinct from *cis*-Golgi membranes in the trafficking pathway. These results pointed to the possibility of simultaneous utilization of two parallel mechanisms for H-Ras transport. One route was the traditional, vesicular path, dependent upon microtubule integrity; the other, a pathway that did not require COP I or COP II vesicles or microtubules.

Some H-Ras accumulates at the ERGIC when the classical pathway is blocked

The identity of the non-Golgi structures that contained H-Ras in nocodazole-treated cells was examined. A clear candidate location was the series of membranes, often termed the ER-Golgi intermediate compartment (ERGIC) [37,38], that bud off the widely dispersed ER exit sites and move toward the Golgi. Microtubule de-polymerization does not prevent traffic from ER to ERGIC, but does interrupt vesicular transport from ERGIC to Golgi. [38]. Thus, at steady-state the bulk of ERGIC membranes are located near the perinuclear *cis*-Golgi, but in nocodazole-treated cells, proteins in the ERGIC reside near the ERES and Golgi mini-stacks. ERGIC membranes are also BFA-resistant, are maintained rather than absorbed during BFA exposure, and even accumulate in a perinuclear mass in cells treated for extended periods with BFA. [38]. Thus, both nocodazole and BFA cause proteins in the conventional trafficking pathway to accumulate in the ERGIC, although the ERGIC membranes are organized in spatially distinct ways by these drugs.

To learn if some H-Ras accumulated on ERGIC membranes in cells in which vesicular transport was impaired, transfected COS-7 cells were treated with cycloheximide for 6 hours, then with nocodazole or BFA for 1.5 hours in the absence of cycloheximide, and finally, were fixed and stained. Antibody to the endogenous ERGIC-53 protein was used to mark the location of ERGIC membranes. In untreated cells, the ERGIC-53 protein was located on a cluster of perinuclear membranes (**Fig. 6, control**). The ERGIC-53-containing membranes

intertwined around the giantin-marked *cis*-Golgi (data not shown), making these structures difficult to resolve in untreated cells. In the minority of untreated cells that showed H-Ras on perinuclear membranes, the newly synthesized H-Ras^{Q61L} protein showed substantial colocalization with the endogenous ERGIC-53 (**Fig. 6, control**).

In agreement with previous reports, in nocodazole-treated cells, the ERGIC-53 antibody stained membranes that had dispersed throughout the cell body (**Fig. 6, + Noc**), while in BFA-treated cells, ERGIC-53 staining appeared on a cluster of perinuclear membranes (**Fig. 6, + BFA**). In cells treated with either nocodazole or BFA, the internal sites where the H-Ras^{Q61L} protein was located colocalized with the ERGIC-53 protein (**Fig. 6**). Once again, H-Ras was also present on the plasma membrane in these nocodazole- or BFA-treated cells. There was also a considerable amount of the newly synthesized H-Ras that was still cytosolic or on the widely dispersed membranes of the ER. These results indicated that some of the newly synthesized H-Ras had entered the traditional trafficking pathway and accumulated in the ERGIC when vesicular transport was impaired.

H-Ras that is in the ERGIC does not access the alternate pathway in nocodazole-treated cells

If H-Ras could move to the plasma membrane via a non-conventional mechanism that originated prior to the Golgi, it might enter this alternate pathway directly from the ER, shortly after interacting with the enzymes that remodel the C-terminus. Alternatively, it might aggregate at ER exit sites and move to the ERGIC via conventional means, but from there disengage and be routed to the cell surface. The proteins that might control H-Ras transport would likely differ at these two locations. The failure of inhibitory Sar1 proteins to prevent H-Ras plasma membrane access suggested that the alternate pathway did not require the proteins found at ER exit sites. Another experiment was designed to test if the H-Ras that

accumulated in the ERGIC upon nocodazole treatment could eventually access the plasma membrane. COS-7 cells were transfected and cycloheximide treated immediately. After 6 hours, cycloheximide was washed out to generate synchronous production of nascent H-Ras proteins, and nocodazole was added at that same time to allow some of these nascent H-Ras proteins to get into, but to impair their exit from, the ERGIC. After 4 hours, when a detectable portion of H-Ras protein accumulated in the ERGIC, a second cycloheximide treatment was applied, to prevent synthesis of additional H-Ras, and the cells, still in the presence of nocodazole, were incubated a further two hours. Thus, only pre-existing H-Ras protein was in the ERGIC, and new protein could not move in. The prediction was that, if H-Ras could be exported from ERGIC via the alternative pathway, it would disappear from the ERGIC, since there would be no new H-Ras supplied from the ER. However, this was not observed. H-Ras was still present in the membranes that aligned with ERGIC-53 even after 2 more hours (**Fig 7, + Noc Chase**). In a control experiment, nocodazole was removed during the second cycloheximide exposure, to now allow vesicular traffic out of the ERGIC. During this nocodazole wash out, the organized, perinuclear structure of the ERGIC quickly re-formed, and H-Ras was observed on many internal, vesicle-like structures that no longer co-aligned with ERGIC-53 (**Fig 7, - Noc Chase**). This confirmed that the H-Ras that had become trapped during the nocodazole treatment had not been damaged and remained competent for transport. Thus H-Ras could rapidly leave the ERGIC if vesicular transport was available, but could leave only very slowly or not at all if that conventional mode of transport was impaired. This result suggests that the two modes of transport are separate, and that H-Ras that reaches the ERGIC can no longer access the alternate route. This supports the

possibility that this alternate pathway for H-Ras transport to the plasma membrane originates directly from the ER.

Discussion

The results here represent the first comprehensive look at modes of transport used to move newly synthesized H-Ras to the plasma membrane. Despite our expectations, conventional vesicular mechanisms could not account for most of the H-Ras that reached the cell surface. Previous work, although focused on the traditional exocytic pathway, had also noted that a significant amount of H-Ras reached the plasma membrane when traditional trafficking pathways were inhibited. [3]. Fluorescence photo-bleaching experiments had also indicated that the H-Ras protein moves from the ER to the Golgi too rapidly to be carried by vesicles. [22].

Those studies, combined with the evidence presented here, lead us to propose that a significant amount of H-Ras uses a non-classical pathway for transportation to the plasma membrane. This model proposes that H-Ras has two pathways by which it can reach the plasma membrane, and that in normal circumstances both routes are used simultaneously. In NIH 3T3 or COS-7 cells, this non-classical pathway appears to be the primary means for plasma membrane access. In yeast, strong genetic evidence supports the idea that the Ras2 protein moves to the cell surface via a non-conventional pathway. [19,39]. This indicates that a non-classical pathway of Ras transport may be conserved in eukaryotes.

This non-classical pathway appears to be initiated at the ER rather than at the Golgi. Comparison of the localizations of H-Ras and Sec13 on ER membranes suggests that very little H-Ras is present at conventional vesicle budding domains of ER export sites. [HZ, unpublished data). In addition, once H-Ras enters the conventional transport pathway and

reaches the ERGIC, it appears to have very limited access to this non-classical pathway. This suggests that the choice of pathway, and mechanism of transport, is determined prior to ER disengagement. H-Ras thus has two distinct means of transport, and, importantly, need not depend on vesicular trafficking for its delivery to the plasma membrane.

Though the two different pathways for H-Ras traffic appear to separate at the ER, it is not yet known what initiates this divergence. One strong candidate for such a mechanism is palmitoylation. [39]. There is existing evidence that, as long as it is non-palmitoylated, farnesylated H-Ras is in a dynamic equilibrium between the cytosol and membranes. [20,22,40]. Current models propose that when H-Ras encounters a membrane-bound palmitoyl transferase and becomes palmitoylated, it can no longer disengage from that membrane. The location at which palmitoylation occurs then defines the point where the palmitoylated H-Ras can join the classical vesicular pathway. [21,22]. Our evidence, that some H-Ras is in the secretory pathway and can reach ERGIC membranes, but, in nocodazole-treated cells, appears unable to leave, provides independent support for this model. The data also bolster the recent report that a palmitoyl transferase may be located on ER membranes [13], and, suggest, additionally, that it may be located on perinuclear ERGIC membranes as well. Our model would propose that regulation of this enzyme's activity might then adjust the amounts of H-Ras that enter each of the two transport pathways. If H-Ras fails to be palmitoylated on the ER, it may be able to use the non-classical pathway to move to the plasma membrane. Experiments with mono-palmitoylated H-Ras mutants are under way to test this prediction. [JM, unpublished data]. An additional prediction, suggested by the large amounts of H-Ras that associated stably with the plasma membrane when vesicular transport was inhibited, is that there are other palmitoyl transferases located at the plasma

membrane. There is already some evidence that supports the possibility of H-Ras palmitoylation on the plasma membrane. A novel H-Ras that is not farnesylated but that retains the native cysteines as sites for acylation, completely avoids the Golgi, yet is targeted to the plasma membrane and becomes palmitoylated. [18,41].

In both yeast and, shown here, mammalian cells, H-Ras transport can function in the apparent absence of traditional vesicles. Neither interruption of outward vesicular movement with nocodazole nor ablation of COP I or COP II vesicles provided any evidence for non-traditional vesicles that might carry H-Ras to the plasma membrane. Thus, the data currently suggest that this non-classical pathway is non-vesicular. The potential lack of a vesicular membrane for interaction of the lipidated C-terminus of H-Ras poses a problem, as the hydrophobic farnesyl (and possibly, palmitoyl) group at the C-terminus would then be exposed to the aqueous phase. This situation would be thermodynamically unfavorable unless the lipid groups were able to fold back against the protein. There is currently no precedent for such a model. An alternative way to prevent exposure of the lipids would be for an escort or chaperone protein to interact with the C-terminus and mask the hydrophobic tail of H-Ras. There is also recent evidence that H-Ras can be found on intracellular particles termed “nanoparticles” or “rasosomes”, that could potentially serve a role in H-Ras transport. [42,43]. Whatever the mechanism, this non-conventional transport of H-Ras is efficient, and delivers H-Ras specifically to the plasma membrane.

Several cancer therapies currently in clinical trials are designed to inhibit membrane binding of Ras proteins. [44]. Our findings that H-Ras may use two pathways to reach the plasma membrane suggests that there is more to learn before this therapeutic strategy will be successful. Of equal importance, the evidence that a significant amount of H-Ras may use

non-vesicular means for movement suggests that proteins that escort or regulate H-Ras trafficking from the ER should be sought. The assays developed in this work provide strategies that will be useful for searching for novel H-Ras partners.

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Figure Legends

Figure 1 H-Ras is not in the perinuclear area in many cells

NIH 3T3 or COS-7 cells were co-transfected with H-Ras^{Q61L} and YFP-GT46 DNAs or transfected with H-Ras^{Q61L} DNA alone. At 18 hours after transfection, cells were fixed and immuno-stained to show the location of the H-Ras protein. Epifluorescence of YFP-GT46 or immunostaining of giantin were used to mark the perinuclear/Golgi area. The strength of the HRas signal in the perinuclear area was assigned a value of “none”, “+” or “++”. Scale bar shows 10 μ m.

Figure 2 H-Ras moves faster than YFP-GT46 to the plasma membrane

NIH-3T3 cells were co-transfected with H-Ras^{Q61L} and YFP-GT46 DNAs.

Cycloheximide was added to the media immediately. Six hours later, that medium was removed and replaced with medium that did not contain cycloheximide. Cells were fixed at different time points after the removal of cycloheximide and stained with antibody to H-Ras. White arrowheads indicate protein that has accumulated in the perinuclear area; white arrows show the plasma membrane. The insets are magnified images of the area enclosed in the box; pink and green arrowheads show vesicles with only H-Ras^{Q61L} (red) or YFP-GT46 (green) respectively; yellow arrowhead shows one of the vesicles that has both H-Ras^{Q61L} and YFP-GT46. Scale bar shows 10 μ m.

Figure 3 BFA or dominant negative Sar1 do not prevent H-Ras from reaching the plasma membrane

A) NIH-3T3 cells were co-transfected with H-Ras^{Q61L} and YFP-GT46 DNAs.

Cycloheximide was added immediately after the transfection and was removed 6 hours later. Brefeldin A was added to the media upon the removal of cycloheximide and, 90 minutes later, cells were fixed and stained with H-Ras antibody. B) NIH-3T3 cells were either co-transfected or sequentially transfected with HA-Sar1^{T39N} and H-

Ras^{Q61L} or HA-Sar1^{T39N} and YFP-GT46 DNAs. At 18 hours after transfection, cells were fixed and stained with H-Ras antibody. Arrowheads show the nuclear envelope and arrows show the plasma membrane. Scale bars equal 10 μ m.

Figure 4 Live cell imaging shows no obvious vesicular traffic of H-Ras to the plasma membrane when BFA is present

NIH-3T3 cells were transfected with GFP-H-Ras^{Q61L} DNA. Cycloheximide was added to the media immediately after the transfection and was removed 6 hours later. BFA was added to the media upon the removal of cycloheximide and live cells were imaged at different time points after the addition. Arrowheads show the perinuclear area. The insets show a higher magnification of the boxed area, with arrows that indicate the stable position of an internal “granule” carrying GFP-H-Ras^{Q61L}. Scale bar shows 10 μ m.

Movie 1 NIH-3T3 cells were transfected with GFP-H-Ras^{Q61L} DNA

Cycloheximide was added to the media immediately after the transfection and was removed 6 hours later. BFA was added to the media upon the removal of cycloheximide and live cells were imaged at 37 degrees starting approximately 30 minutes later, when protein had been synthesized in amounts sufficient to be visible.

Figure 5 H-Ras still reaches the plasma membrane when nocodazole is present

NIH-3T3 cells were transfected with H-Ras^{Q61L} or YFP-GT46 cDNAs. Cycloheximide was added to the media immediately after the transfection and was removed 6 hours later. Nocodazole was added to the media upon the removal of cycloheximide and after 90 minutes (120 minutes for YFP-GT46, right panel, first row) cells were fixed and immuno-stained with antibody for giantin and, in the H-Ras-transfected cells (the left and middle columns), antibody for H-Ras. Arrows indicate the plasma membrane.

Merge panels are enlargements of the boxed areas, with arrowheads that indicate two separate structures, one containing H-Ras^{Q61L} or YFP-GT46 (green), the other containing giantin (red). Scale bar shows 10µm.

Figure 6 When nocodazole or BFA is present, a portion of H-Ras accumulates in the ERGIC

COS-7 cells were transfected with H-Ras^{Q61L} cDNA. Cycloheximide was added to the media immediately after the transfection and was removed 6 hours later. Nocodazole or BFA were added to the media upon the removal of cycloheximide and, after 90 minutes, cells were fixed and stained with antibodies for H-Ras and ERGIC-53. Arrows show the plasma membrane; arrowheads show the colocalization of H-Ras and ERGIC-53. Scale bar shows 10µm.

Figure 7 H-Ras does not leave the ERGIC in the presence of nocodazole. COS-7 cells were transfected with H-Ras^{Q61L} DNA

Cycloheximide was added to the media immediately after the transfection and was removed 6 hours after the transfection. Nocodazole was added to the media upon the removal of cycloheximide and after 2 hours cycloheximide was added again to the media while nocodazole was either washed out (-Noc) or still kept in the media (+Noc). After another 2 hours, cells were fixed and immuno-stained. Arrows show the plasma membrane; arrowheads show the ERGIC membrane. Scale bar shows 10µm.

Table Legends

Table 1 HRas perinuclear distribution is distinct from the pattern of a protein in the traditional vesicular trafficking pathway

NIH 3T3 cells and Cos-7 cells were co-transfected with YFP-GT46 and one of the H-Ras DNAs was indicated. After 18 hours, the cells were fixed and immuno-stained for HRas.

Approximately 100 cells were examined and the strength of the HRas signal in the perinuclear area was assigned values of “none”, “+”, or “++”. Values are the averages and standard deviations of the number of cells from two (HRas) or four (YFP-GT46) independent experiments. Monomeric GFP is abbreviated as mGFP and “N.d.” means not determined.

Figures

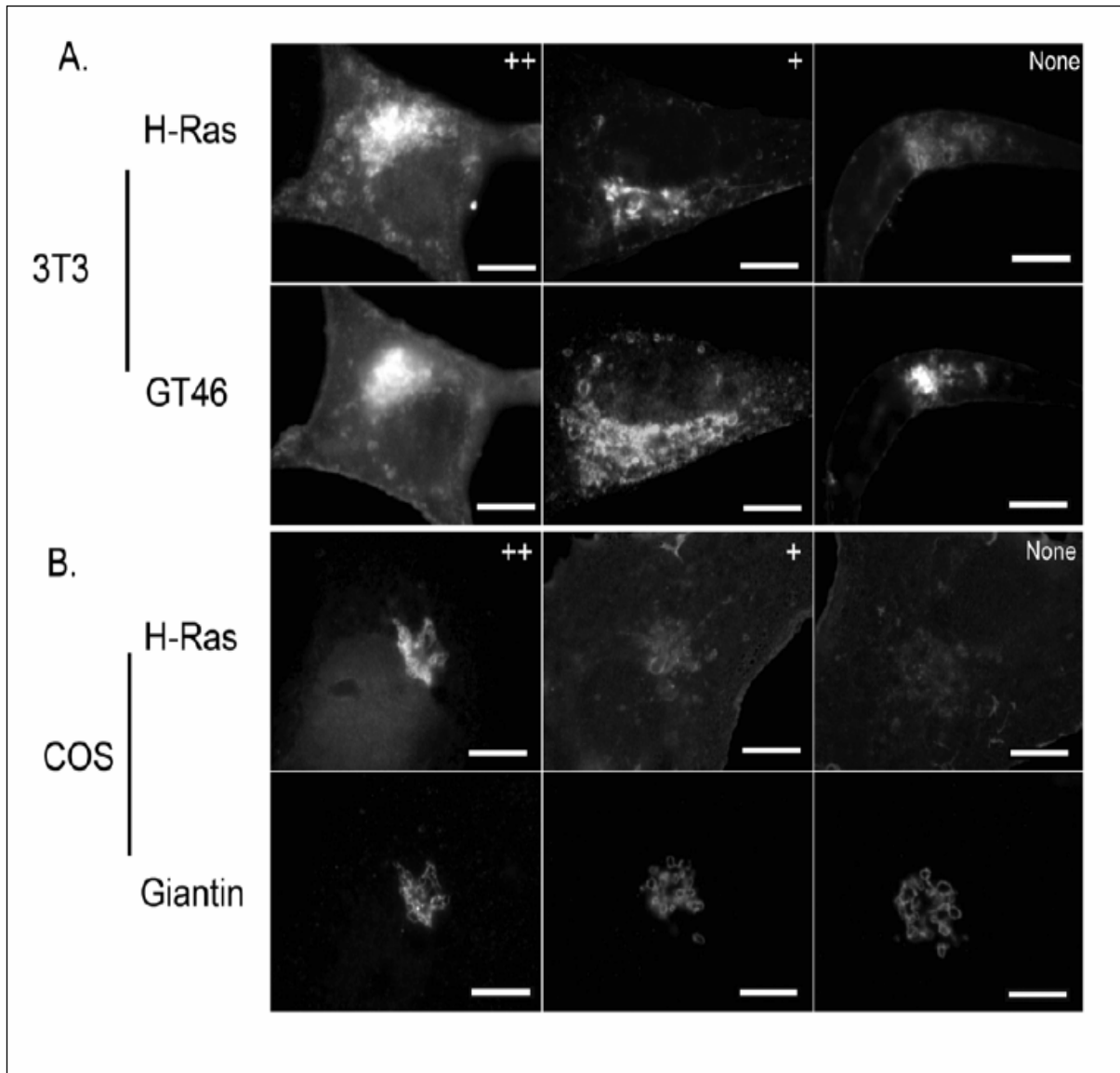


Figure 1 H-Ras is not in the perinuclear area in many cells

Table1 HRas perinuclear distribution is distinct from the pattern of a protein in the traditional vesicular trafficking pathway

		Cell Type					
		NIH-3T3			COS-7		
Fluorescence in perinuclear area		++	+	None	++	+	None
D N A T Y P E	YFP-GT46	92 ± 9	4 ± 3	5 ± 5	94 ± 3	2 ± 1	3 ± 2
	H-Ras ^{wt}	6 ± 5	10 ± 6	83 ± 1	4 ± 1	3 ± 2	93 ± 3
	H-Ras ^{Q61L}	1 ± 1	5 ± 4	94 ± 4	7 ± 4	9 ± 4	83 ± 8
	GFP-H-Ras ^{wt}	45 ± 14	23 ± 2	32 ± 15	50 ± 2	17 ± 3	33 ± 1
	GFP-H-Ras ^{Q61L}	59 ± 5	6 ± 8	36 ± 3	59 ± 12	14 ± 4	28 ± 8
	mGFP-H-Ras ^{Q61L}	23 ± 2	23 ± 2	51 ± 5	n.d.	n.d.	n.d.

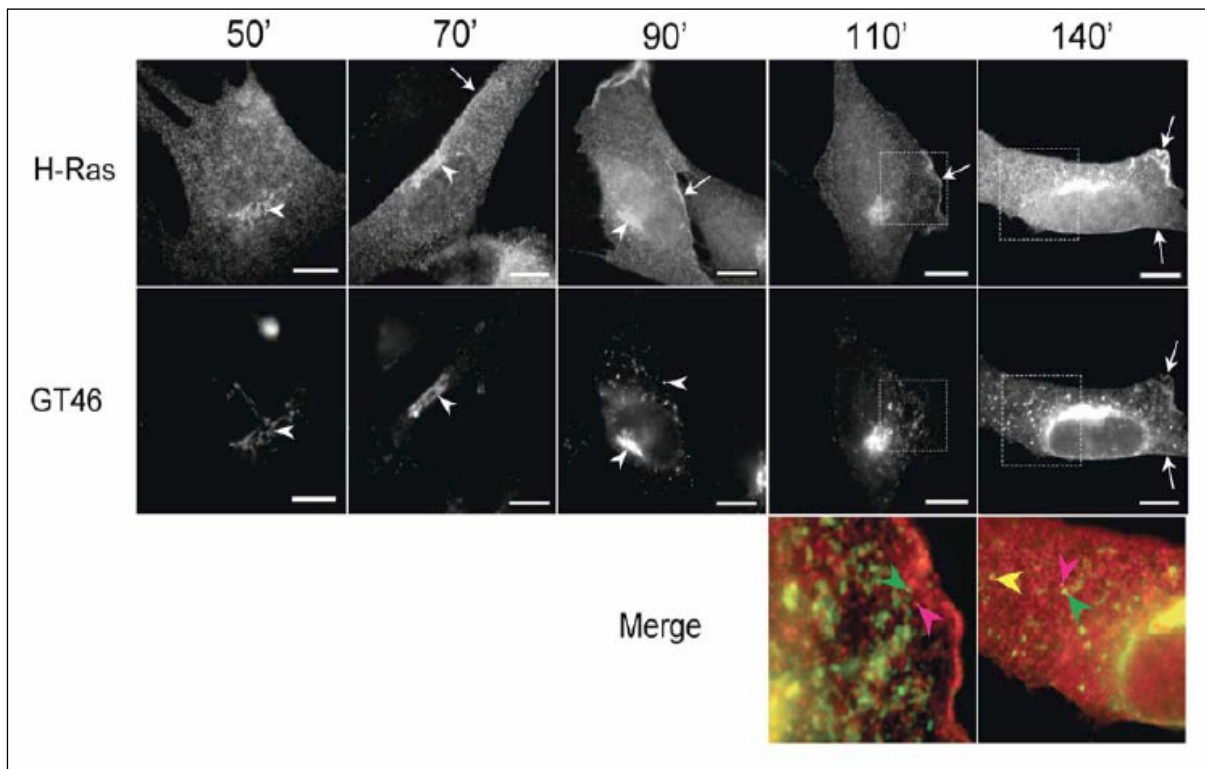


Figure 2 H-Ras moves faster than YFP-GT46 to the plasma membrane

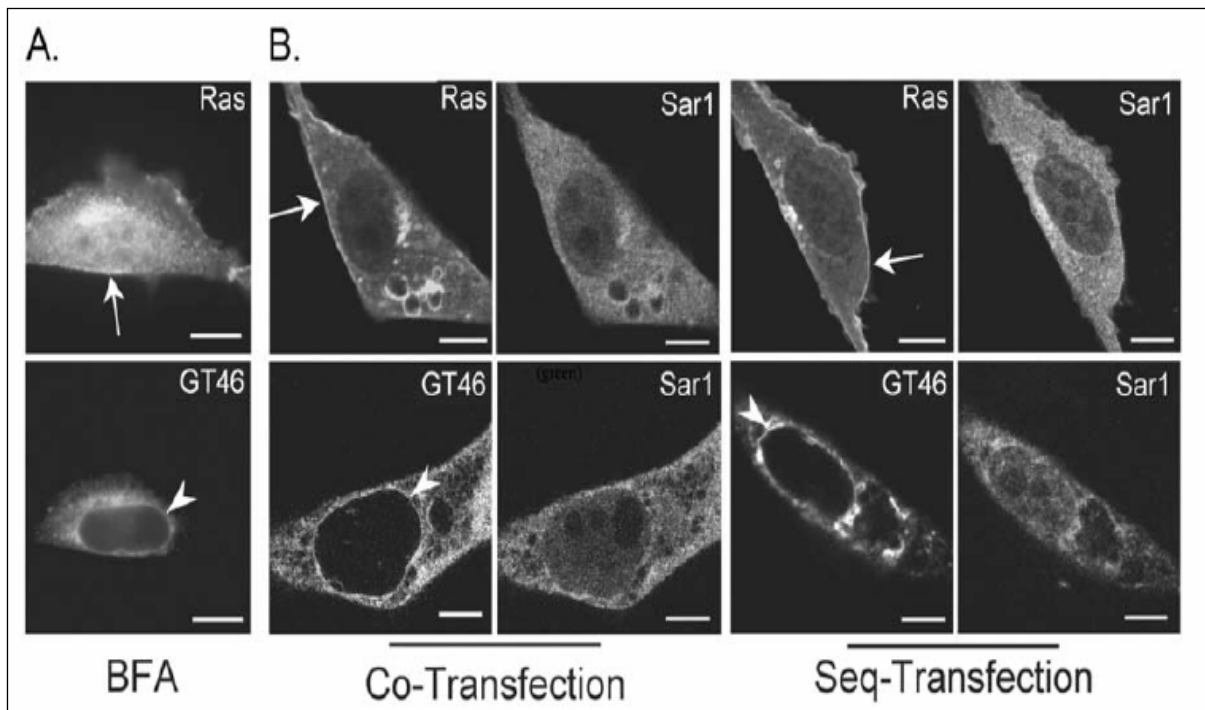


Figure 3 BFA or dominant negative Sar1 do not prevent H-Ras from reaching the plasma membrane

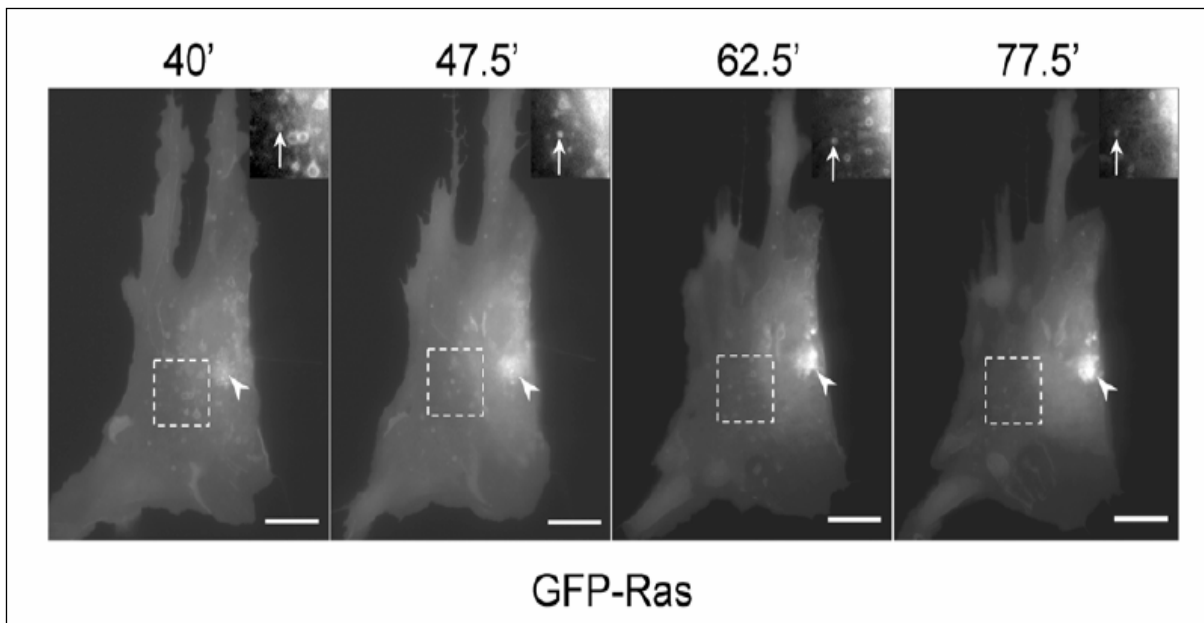
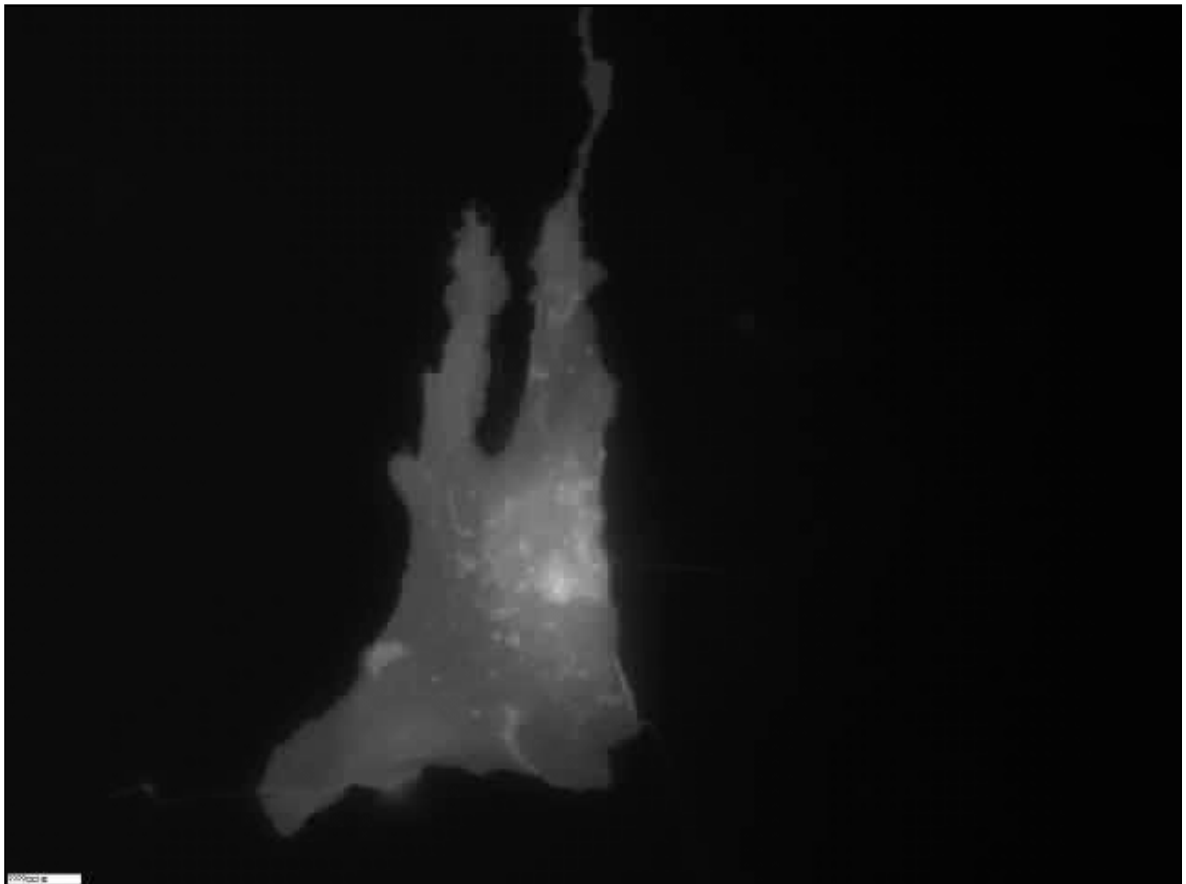


Figure 4 Live cell imaging shows no obvious vesicular traffic of H-Ras to the plasma membrane when BFA is present



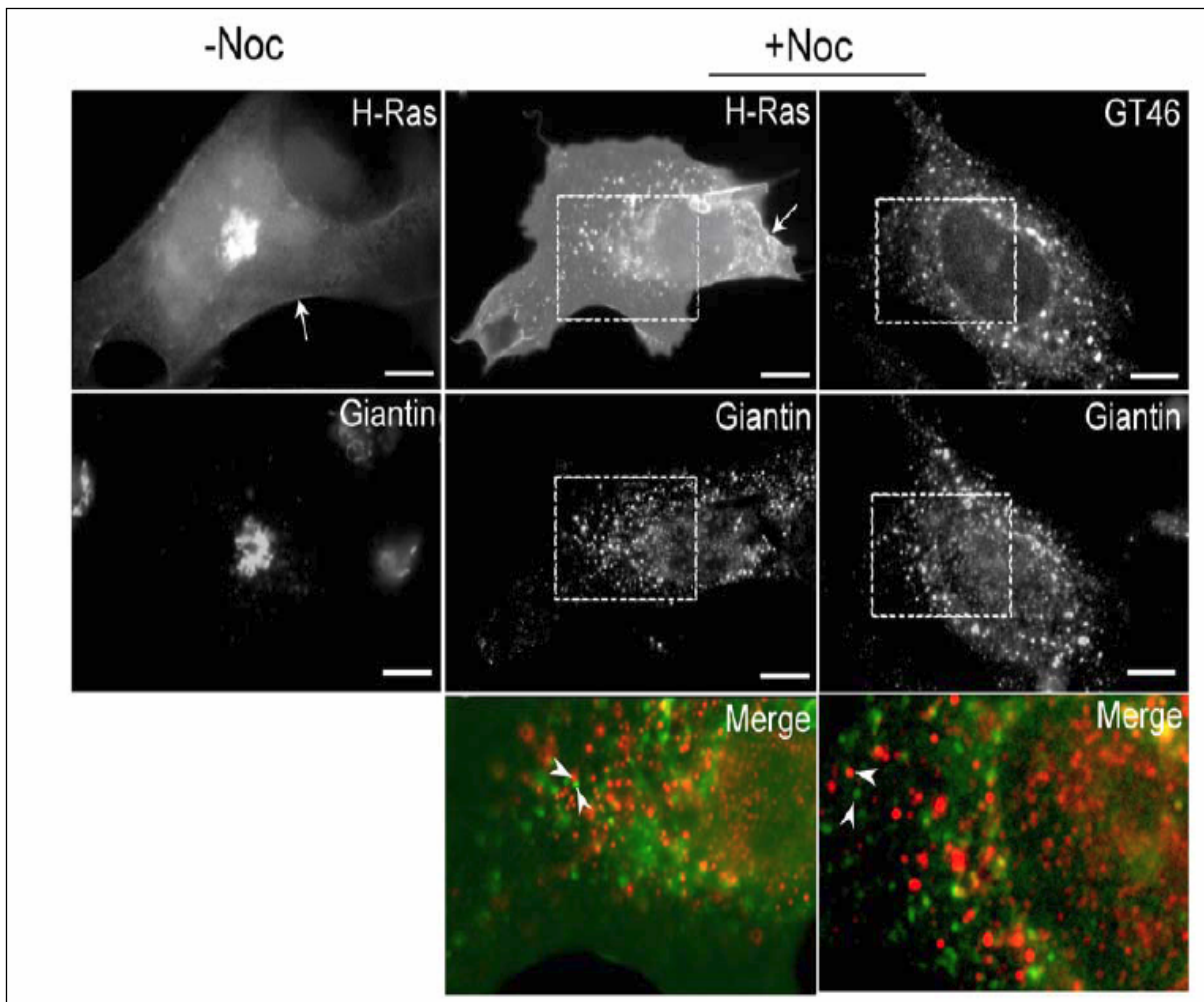


Figure 5 H-Ras still reaches the plasma membrane when nocodazole is present

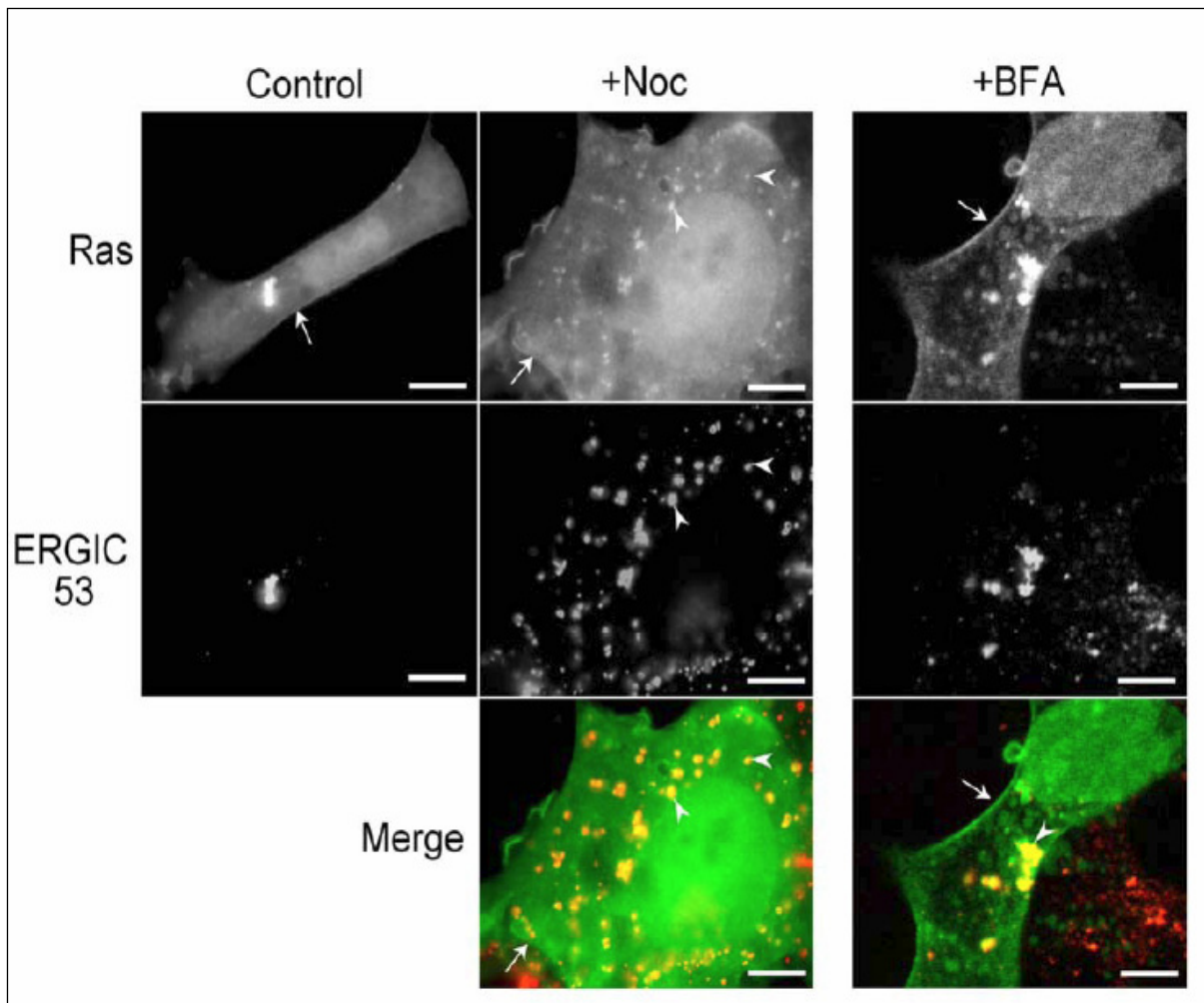


Figure 6 When nocodazole or BFA is present, a portion of H-Ras accumulates in the ERGIC

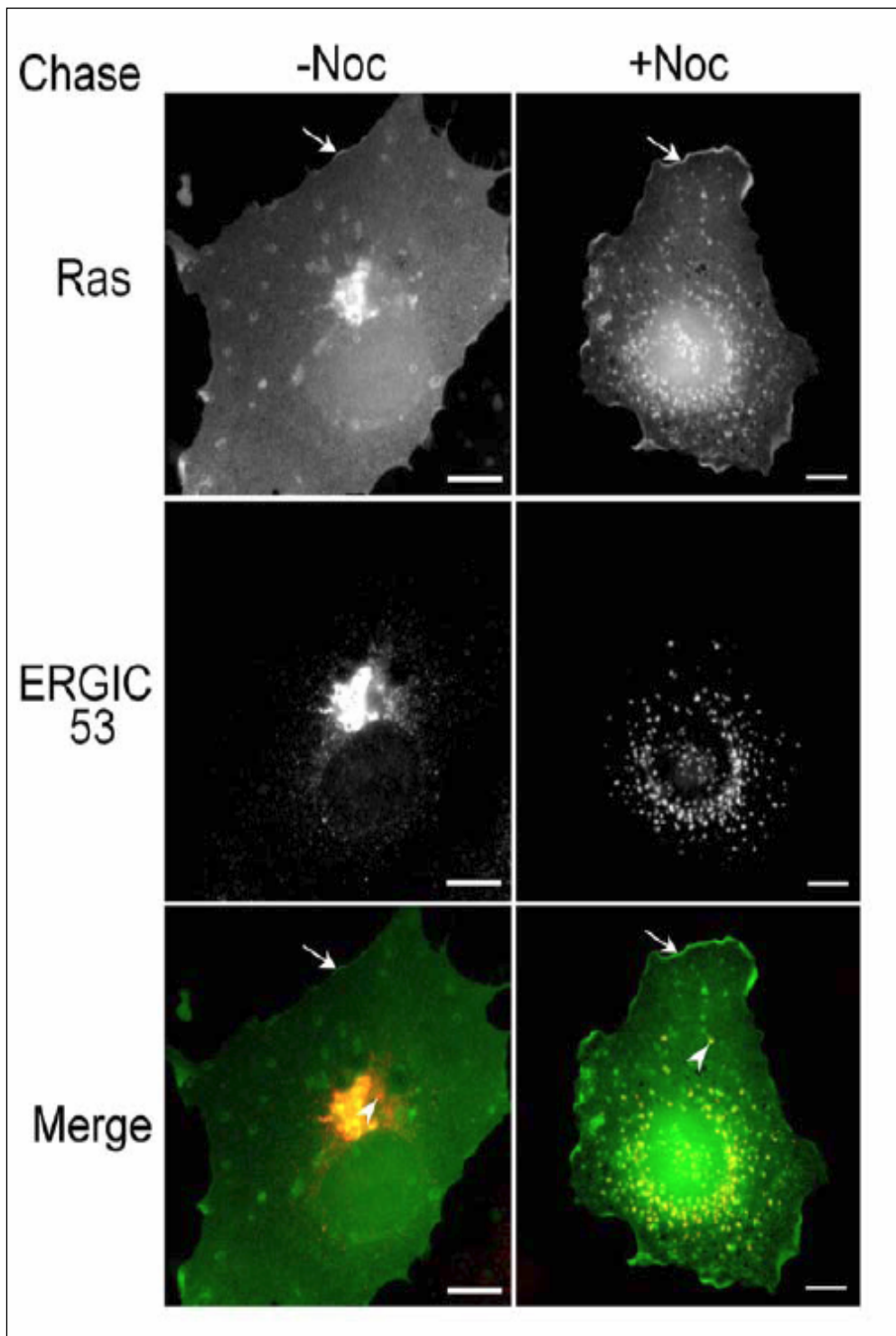


Figure 7 H-Ras does not leave the ERGIC in the presence of nocodazole. COS-7 cells were transfected with H-Ras^{Q61L} DNA

Trafficking requires conservation of the HRas hypervariable region

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Running Title: HRas hypervariable region determines mode of trafficking

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Abstract

Recent studies indicate that multiple exocytosis pathways are used by HRas to reach the plasma membrane in yeasts and mammals. When Ras is attached to GFP, more instances of vesicular trafficking are seen, but in the absence of GFP, the majority of Ras bypasses the Golgi on its way to the plasma membrane. In this study we have further established that GFP emphasizes the vesicular trafficking pathway in various polarized epithelial cell types, such as MDCK (Madine Canine Kidney Cells) and HeLa (Henrietta Lacks cells). To date, we have seen a Golgi-independent-trafficking mechanism used predominantly in NIH 3T3 cells and Cos-7 cells. To more clearly understand Golgi-independent trafficking mechanisms, we explore which properties of HRas are required for the non-vesicular trafficking pathway. We have found that all palmitate lipid modifications as well as the entire hypervariable region are needed for HRas localization at the plasma membrane via the Golgi-independent trafficking mechanism.

Introduction

Ras is a member of the small GTPase family that plays an essential role in cellular function. It is often described as a molecular switch that exists in two states—GTP bound (active) and GDP bound (inactive). When it is activated, Ras is able to interact with multiple down stream effectors that ultimately result in cell proliferation, differentiation, and apoptosis. While the four major isoforms of Ras—HRas, NRas, KRas A, and KRas B—are highly conserved with each other in the conserved region, their isoforms differ in their hypervariable regions at the carboxy terminus (C-terminus).

In order to become fully functional as a molecular switch, newly synthesized Ras goes through a series of posttranslational modifications. The posttranslational modification takes place at the final four residues of Ras often described as the *CAAX* box (*C* = cysteine = cys 186; *AA* = aliphatic amino acids; and *X* = any amino acid). To begin the posttranslational modification, C of the CAAX box is modified by the enzyme farnesyl transferase. Then the farnesylated Ras binds to the cytoplasmic leaflet of the Endoplasmic Reticulum (ER) where Ras Converting Enzyme 1 (RCE 1) cleaves the -AAX of the CAAX box. [1, 2] The newly exposed cysteine is then modified by the Isoprenyl Cysteine Carboxy Methyl Transferase (Icmt; methyl transferase).[3-5] At this point KRas, with its positively charged polylysine residues, localizes to the plasma membrane by an unknown mechanism, but NRas and HRas require additional modifications before membrane localization. [2] The enzyme Palmitoyl Transferase attaches the palmitate lipid groups onto the cysteines just upstream of the CAAX box. NRas receives a single palmitate at position 181, whereas HRas receives double palmitates at positions 181 and 184. [6, 7] These palmitates serve as membrane anchors that

adhere Ras to the cytoplasmic leaflet of the plasma membrane where Ras acts as a molecular switch. [8-10]

Early models show that in order for Ras to act as a molecular switch, it must be localized to the plasma membrane. [11] In fact, mutations that prevent Ras from localizing to the plasma membrane show significant decreases in Ras-mediated oncogenic activity. [12] On the other hand, Ras that is localized on endomembranous organelles, such as the Endoplasmic Reticulum (ER), is thought to be a protein that is in transit before it localizes to the plasma membrane via vesicles. The process by which Ras travels out to the plasma membrane in vesicles is known as the *vesicular trafficking mechanism*. For HRas, vesicular trafficking begins when palmitoylated Ras is released into the cytoplasmic leaflet of the ER. [1, 13] In the ER, vesicle formation begins when a small GTPase—Sec-related and Ras-related protein 1 (Sar 1)—is activated by GTP binding. [14] Activation of Sar1 leads to aggregation of the COP II complex of proteins around the ER Exit Sites (ERES). [15-17] Before reaching the Golgi, COP II-coated vesicles will first merge into a vesiculo-tubular intermediate structure (ER-Golgi-Intermediate-Compartment, ERGIC). [18] From here, cargo-containing COP II vesicles continue their trafficking to the Golgi and, ultimately, to the plasma membrane.[19] In some cases, vesicles are transported back to the ER from Golgi, This kind of retrograde trafficking is regulated by the COP I complex. [19]

An antifungal reagent, Brefeldin A (BFA), inhibits the COP I complex from binding to the ER membrane but allows retrograde trafficking to continue. Continued trafficking in one direction causes Golgi membranes and proteins to rapidly regress into the ER and ultimately results in the collapse of the Golgi.

Several interesting, recent studies show that even in the absence of Golgi, HRas anterograde trafficking to the plasma membrane continues, suggesting a Golgi-independent mode of trafficking for HRas. Some have even suggested the existence of nanoparticles or the possibility of Ras oligomer formation. [20, 21] Further, this Golgi-independent mechanism seems to also be vesicle-independent. [22-24] Finally, a potassium-channel subunit, KchIP, and KRas also seem to localize to the plasma membrane in a Golgi-independent manner. [25, 26]

In this study, we examine the roles that palmitoylation may play in Golgi-independent and vesicle-independent trafficking. We also explore whether any of the non-palmitoylated residues upstream of the CAAX box are key players in Golgi-independent HRas trafficking.

Materials and Methods

DNA and plasmids

HRas C181S, HRas C184S, and HRas Q61L were expressed in PcDNA3 plasmid (Invitrogen). GFP-HRas Q61L is expressed in pEGFP-C3 vector. GFP delta 17 and GFP delta 19 were gifts from Dr. Ian Prior. Briefly, for delta 17 mutants, 7 alanine residues were inserted just upstream of the CAAX box. (HKLRKLNPPDESGPGCMSCKCVLS to HKLRKLNAAAAAAGCMSCKCVLS). For delta 19 mutants, 7 alanine residues were inserted further upstream from the CAAX box (HKLRKLNPPDESGPGCMSCK to AAAAAAAPPDESGPGCMSCK). Both mutants were expressed in GFP-C1 vector. YFP-GT46, mGFP-HRas Q61L, and mGFP-HRas wildtype (wt) were gifts from Dr. Anne Kenworthy.

Cell culture and transfection

NIH3T3, HeLa, and Cos-7 cells were cultured in 10% CO₂ in Dulbecco's Modified Eagle's Medium (DMEM from GIBCO-BRL). DMEM also contained 10% calf serum (GIBCO). One percent each of sodium pyruvate (GIBCO), L-glutamine (GIBCO), antibiotics

(GIBCO), and Fungazone (Sigma) were added. MDCK cells were cultured at 10% CO₂ in DMEM, which also contained 10% fetal bovine serum (GIBCO) and 1% each of L-glutamine, sodium pyruvate, antibiotics, and Fungazone. To seed the cells, pre-washed glass coverslips were placed in 12 well-tissue-culture plates and were coated with a mixture of polylysine and fibronectin for at least 2 hours at 37°C. Twenty-four hours before DNA transfection, NIH3T3 cells were placed on these coverslips at a density of approximately 10⁵–10⁶ cells per well.

Transfection was done using Effectene transfection reagent (Qiagen) following the manufacturer's protocol. For experiments where double transfection was needed, DNA were combined (0.15µg of each type of DNA per well) before transfection.

Cell counts

NIH 3T3, HeLa, MDCK, and Cos-7 cells were co-transfected with YFP-GT46 and HRas wt or HRas Q61L mutant. YFP-GT46 is an artificial, secretory protein that consists of the Yellow Fluorescent Protein (YFP), the N-glycosylation site, the transmembrane domain of the LDL receptor, and the cytoplasmic tail of CD 46. After 24 hours, cells were immunostained with HRas antibody. Approximately 150 cells expressing YFP-GT46 in the perinuclear region were counted. Of these same cells, whether HRas was present in the same perinuclear area were noted. At least two individual counts were performed and the data were analyzed using ABI Prism software.

Brefeldin A, Nocodazole, and Cycloheximide treatments

For cycloheximide treatments, NIH 3T3 cells were treated with cycloheximide (50µg/ml) immediately following the transfection. The cells were incubated at 37°C for 6 hours, then cycloheximide was washed out with phosphate buffered saline (PBS). Cells in complete

medium were then treated for 4 hours (unless otherwise noted) with either BFA (10 μ g/ml) or nocodazole (20 μ g/ml). Cells were then fixed and stained for immunofluorescence.

Immunofluorescence

Following transfection and the pharmacological treatments described above, NIH 3T3 cells were fixed with 4% paraformaldehyde in PBS at room temperature for 1 hour. Cells were then permeabilized with either 100% methanol at -20°C for 1 minute or 0.2% Triton X-100 on ice for 5 minutes. Cells were then quenched twice (15 minutes each) with 50mM NH_4Cl in PBS before being blocked. For the blocking step, cells were incubated in blocking buffer (2% horse serum, 0.4% BSA in PBS) at room temperature for 30 minutes. Immediately after blocking, the cells were incubated in primary antibodies diluted in blocking buffer at room temperature for 1 hour (HRas 238 Rat monoclonal IgG, 1:400 dilution, Santa Cruz; giantin anti-rabbit polyclonal antibody, 1:800, Covance; TRAP anti-rabbit 1:500, Nicchita). Then the cells were incubated in secondary antibodies (Alexa-Fluor[®] donkey-anti-rat, 594 1:800, Alexa-Fluor[®] goat-anti rat 488 1:800, Alexa-Fluor[®] goat anti-rabbit 594, 1:800; all secondary antibodies were purchased from Molecular Probes) diluted in blocker solution for an additional hour in the dark. For phalloidin labeled experiments, the cells were incubated in Alexa-Fluor[®] 594-conjugated phalloidin (Molecular Probes, 1:200 dilution in blocking buffer) for 20 minutes in the dark. After labeling with secondary antibodies, the coverslips containing cells were washed in PBS and distilled water before being mounted on glass slides with mounting media (Vectashield). Finally, the coverslips were rimmed with nail polish.

Microscopy

All cells were examined and photos were taken with Leica DMIRE2 microscope with a numerical aperture of 100x. Images were first captured in black and white, then colors were added using Open Lab 3.5.1 software. The images were then exported to Adobe Photoshop 7 to equalize intensity of both images and then merged. Unless noted in the figure legends, all images were taken in Z-stack mode.

Results

GFP-HRas expression in multiple cell types

HRas localizes on endomembranous structures such as the Golgi and the ER in addition to the plasma membrane. To study HRas localization, green fluorescent proteins (GFP) are often attached to the amino terminus of HRas to make a GFP-HRas—an HRas chimeric protein. As an HRas chimera, GFP acts as a reporter and indicates where its fusion partner is located, even on membrane structures. Although in these situations GFP is a great reporter, effects that the approximately 50 kDa GFP might have on HRas localization and trafficking patterns have not yet been closely examined because when GFP is expressed without fusion to HRas, GFP is evenly distributed in the cytosol and absent from membrane structures.

When GFP is attached to the HRas N-terminus, GFP-HRas will localize at the plasma membrane and other endomembranes. In fact, our studies show that GFP-HRas emphasizes perinuclear accumulation of HRas more than their non-GFP tagged counterparts. (Chapter 2) When NIH 3T3 cells were transfected with GFP-HRas, there were frequently strong localizations at the plasma membrane and at the structure that appears to be Golgi near the nucleus. **(Figure 1, Table 1)** At first we entertained the possibility that these accumulations are directly related to GFP-HRas over-expression. In high concentrations, GFP has the ability to form dimers, so it was possible that the GFP of the GFP-HRas could bind with one another

to form dimers. Formation of GFP dimers might change the rate of HRas trafficking to the plasma membrane because of increases in the size of HRas. When monomeric GFP (mGFP), a GFP mutant that is unable to form dimers, is attached to HRas and expressed in NIH 3T3 cells, even though mGFP-HRas was able to reach the plasma membrane, there was a decrease in the percentage of cells showing the perinuclear accumulation. On average, 60–70% of cells expressing GFP-HRas61L showed Golgi-like accumulation near the nucleus. In contrast, monomeric GFP version of the same HRas (mGFP HRas Q61L) accumulated near the nucleus in only about 25% of the cells. Although mGFP significantly decreased HRas accumulation in the perinuclear area, mGFP HRas is still seen significantly more frequently in the perinuclear area than HRas Q61L (<10%) which does not contain any reporters.

(Figure 1, Table 1)

To identify the perinuclear structure in HRas-expressing cells, NIH 3T3 cells were co-transfected with HRas and YFP-GT46, a artificial, secretory, chimeric protein that is known to use the conventional trafficking mechanism. YFP-GT46 consists of the yellow fluorescent protein (YFP), an N-glycosylation site, the transmembrane domain of LDL receptor, and the cytoplasmic tail of CD 46. [27] When we co-transfected NIH 3T3 cells with YFP-GT46, this protein marked the entire conventional trafficking pathway and the perinuclear Golgi region. At 24 hours after co-transfection, most of YFP-GT64 was localized at the perinuclear region as well as at the plasma membrane. To verify that these perinuclear regions are, in fact, Golgi, giantin (a Golgi-resident protein), was used as a marker to visualize the cis-Golgi structure. As expected, there were strong colocalizations between giantin and YFP-GT46. (data not shown)

Recently, Gomez et al. described restricted HRas accumulation at the Golgi apparatus in the absence of GFP tags in CHO-K1 cells. [28] To see if the lack of HRas accumulation at the Golgi is observable in other cell types, the experiments reported above were repeated with four cell types, MDCK, HeLa, NIH 3T3, and Cos-7. When expressed, GFP HRas also showed strong Golgi accumulation in MDCK and HeLa cells. When cells were co-transfected with YFP GT 46 and untagged HRas, the majority of cells of all four cell types showed HRas only at the plasma membrane. (**Figure 2; Table 2**)

We examined whether the GTP- or GDP-bound state of HRas changed its subcellular localization patterns. An oncogenic mutant form of HRas (HRas Q61L) or a cellular form of HRas (HRas wt) was co-transfected with YFP-GT46. Although YFP-GT46 showed strong accumulation both at the Golgi and at the plasma membrane as before, there were no significant differences between the oncogenic or cellular types of HRas colocalizing with perinuclear YFP-GT46. (**Table 1, Table 2**) These results suggest two interesting possibilities. First, GFP changes HRas trafficking by decreasing the rate of movement and decreasing HRas trafficking efficiency toward the plasma membrane through the Golgi. GFP dramatically slows the HRas trafficking mechanism. If GFP changes HRas trafficking efficiency and the rate of movement through the Golgi, it follows that all HRas must pass through the Golgi.

However, a second possibility was considered: how does naturally occurring HRas traffic out to the plasma membrane in the absence of Golgi. In our previous study (Chapter 2), we observed that HRas trafficked out to the plasma membrane in a Golgi-independent manner, while GFP-tagged HRas prefers Golgi-dependent trafficking. In other words, non-GFP-tagged HRas prefers the Golgi-independent trafficking mechanism more than GFP-tagged

HRas does. It is not yet known if more than one Golgi-independent trafficking mechanism exists.

HRas plasma membrane localization with BFA

To investigate these possibilities further, NIH 3T3 cells were co-transfected with HRas Q61L and YFP-GT46. To insure that only outward-bound HRas are visualized, cells were treated with cycloheximide, a pharmacological reagent that inhibits translation. If cycloheximide is added immediately after transfection, cells may accumulate HRas RNA, but no HRas protein will be translated or trafficked. To test whether Golgi is an essential component in HRas trafficking, YFP-GT46 and HRas Q61L transfected NIH 3T3 cells were treated with cycloheximide for 6 hours. Then they were washed with PBS and treated with Brefeldin A (BFA). BFA is a pharmacological reagent that collapses the Golgi and prevents vesicular transport. As expected, when treated with BFA, YFP-GT46 was absent from the plasma membrane in NIH 3T3 cells (**Figure 5**). YFP-GT46 proteins were predominantly located on the internal membranes and on the circumference of the nucleus. This is consistent with proteins located on the ER.

On the other hand, most HRas Q61L was localized only at the plasma membrane. (**Figure 3**) In our previous study (Chapter 2), we noticed that HRas can reach the plasma membrane much faster than YFP-GT46 even when protein trafficking to the plasma membrane was synchronized (HRas = 20 minutes; YFP GT46= 2 hours). Even the small punctate structures in transit in the cytosol showed very little, if any, colocalization between HRas and YFP-GT46.

Mono-palmitoylated HRas does not accumulate in the Golgi

It has been well established that HRas palmitoylation is essential for its plasma membrane localization. Even though some isoforms of Ras are not dependent on palmitoylation for plasma membrane localization (e.g., KRas), further study was needed to determine whether 1) mono-palmitoylated HRas is still able to localize to the plasma membrane and 2) Golgi-independent exocytosis is available to HRas with one palmitate. Roy et al. described mono-palmitoylated HRas being localized at the plasma membrane as well as at the Golgi apparatus. [29] To confirm these findings, we co-transfected HRas with either C181S or C184S and with YFP-GT 46. As expected, approximately 98% of the cells expressing YFP-GT46, showed very strong Golgi and plasma membrane localizations. In addition to the study done by Hancock et al., very few (<10%) NIH 3T3 cells with HRas C181S or C184S (**Figure 4**) showed strong Golgi localization even though both mutants illuminated the plasma membrane very clearly. (**Figure 5, Figure 6**) It is likely that this discrepancy between our findings and other published data may be due to the attachment of GFP on the N-terminus of the mono-palmitoylated HRas proteins used in other studies. As previously mentioned, adding GFP to HRas enhances protein accumulation in the Golgi and thus emphasizes the usage of Golgi-dependent trafficking mechanism.

Palmitoylation requirements for Golgi-independent trafficking

To further investigate whether the Golgi-independent trafficking mechanism is available to mono-palmitoylated HRas, NIH 3T3 cells were co-transfected with YFP-GT46 and either C181S HRas or C184S HRas. Immediately following the transfection, cells were exposed to cycloheximide for 6 hours to synchronize protein trafficking then they were treated with BFA to collapse the Golgi. To our surprise, both HRas C181S and HRas C184S failed to localize to the plasma membrane (**Figure 3**), but HRas Q61L reached the plasma membrane

as expected. (**Figure 5; Figure 6**) We also noted that neither HRas Q61L, HRas C181S, or HRas C184S colocalized with YFP-GT46 when vesicles in transit were examined. To consider the possibility that these HRas mutants retreated back into the ER in the absence of Golgi, the experiments were repeated and cells were immuno-labeled with an ER-resident marker (α trap). Even in the regions near the nucleus where the ER is most densely packed, no colocalization between HRas palmitoylation mutants and ER-resident protein was observed. In the presence of BFA, both mutants appear to be mostly cytosolic or in tiny punctate structures. (**Figure 5**)

Because mono-palmitoylated HRas was clearly absent from the plasma membrane but not retained in the ER, we repeated the experiment using nocodazole, which destroys the microtubular tracks, to see if mono-palmitoylated HRas were trapped in the vesicles. Once formed, vesicles usually travel along the microtubular tracks to move within a cell and to deliver cargo proteins to the plasma membrane. New tracks of microtubules are constantly being synthesized while old tracks are being destroyed. An overall net growth of microtubules occurs when GTP-bound tubulins are attached to the terminal GDP-bound tubulin. When GTP hydrolysis is much faster than the addition of new tubulins, microtubules begin to shrink and are eventually destroyed. Nocodazole, a pharmacological reagent, is commonly used to depolymerize microtubules in these ways.

When HRas C181S or HRas C184S mutants were co-expressed in NIH 3T3 cells along with YFP-GT46, all of the constructs were easily visible on the plasma membrane, and only YFP-GT46 was aggregated in the Golgi region. However, when the experiment was repeated and cells were treated with cycloheximide and nocodazole, neither mutant illuminated the plasma membrane. It seems that when vesicular trafficking is inhibited, HRas C181S and

HRas C184S are largely cytosolic or in small clusters much like the ones observed during BFA treatments. (**Figure 6**) Likewise, both BFA and nocodazole treatments disperse YFP-GT46 around the nuclear envelop as well as small, dot-like structures in the cytosol, but they are not co-aligned to HRas dots.

Non-palmitoylated residues and HRas plasma membrane localization

The trafficking experiments reported here that were conducted with mono-palmitoylated HRas demonstrate that mono-palmitoylation alone is insufficient to localize HRas to the plasma membrane through Golgi-independent pathways. However, not only are each of the final 10 amino acid residues of HRas sufficiently lipid-modified to reach the plasma membrane, HRas can do so through the Golgi-independent mechanisms. To investigate whether non-palmitoylated residues between the two palmitate groups are needed for Golgi-independent trafficking, we conducted an experiment with a new HRas mutant—HRas CDQCE. As the name indicates, the HRas CDQCE mutant has the C-terminus amino acid sequence —CDQCECVLS, as opposed to cellular HRas which has the sequence —CMSCKCVLS. This mutant contains all of the lipid modification sites but the residues in between the palmitate groups have been changed. When the CDQCE mutant was transfected into the NIH 3T3 cells, there was a small accumulation of HRas in the Golgi, but the HRas CDQCE proteins were largely absent from the plasma membrane. (**Figure 7**) To eliminate the possibility that this mutant localizes to the plasma membrane at a much slower pace than their cellular counterparts, proteins were synthesized and trafficked for up to 48 hours. Even at 48 hours post-transfection, HRas CDQCE was unable to reach the plasma membrane, whereas HRas wt was able to reach the plasma membrane in less than one hour. We also

noted that the trafficking of and localization (i.e. internal) of GTP-bound HRas 61L CDQCE mutant was similar to that of the wt GDP bound form (data not shown).

Conservation of the amino acids further upstream of the CAAX box is also required for Golgi-independent HRas trafficking

After having observed that each of the terminal 10 amino acids seem to contribute to HRas trafficking and plasma membrane localization, we expanded the study to explore how other parts of the hypervariable region serve as determining factors for HRas trafficking and localization. To address this question, we obtained two additional HRas mutants from Dr. Ian Prior's lab. The first of the two mutants, GFP delta 17, contains alanines that were substituted for residues 166 through 172 (—HKLRKLNAAAAAAGCMSCKCVLS). Likewise, the second mutant, GFP delta 19, also contains seven alanine substitutions that span from residue 173 through residue 189. **(Figure 8)** When each of these mutants was expressed in NIH 3T3 cells, they both showed a very strong localization of HRas at the plasma membrane and at the Golgi, like HRas wt or HRas Q61L. This perinuclear accumulation was expected as both proteins are GFP chimeras.

To see whether these alanine mutants reach the plasma membrane through a Golgi-independent mechanism, transfected cells were first treated with cycloheximide and BFA, then co-immuno-labeled with giantin. As expected, giantin-labeled Golgi structures were completely destroyed in the presence of BFA. Because both of the alanine mutants contain the final 10 amino acids in the HRas tail, it seemed reasonable that both the GFP delta 17 and the GFP delta 19 mutants would exhibit trafficking behavior that is similar to HRas 61 and GFP HRas tail. However, it seemed that neither the GFP delta 17 nor the GFP delta 19 mutants were able to reach the plasma membrane in the presence of BFA, much like HRas C181S and HRas C184S constructs. On rare occasions, some transfected cells did show

minute amounts of alanine mutants on plasma membranes, but these were only apparent on cellular ruffles. To confirm that the delta mutants did not localize at the plasma membrane, experiments were repeated and GFP delta 17 and GFP delta 19 mutants were co-immunolabeled with Alexa-Fluor® 594-conjugated phalloidin, a toxin found in a specific type of mushroom that binds to the F-actin and clearly indicates plasma membranes. As expected, the delta 17 and delta 19 proteins did not show any colocalization with phalloidin in NIH 3T3 cells. (**Figure 9**)

Discussion

Although HRas uses the classical, Golgi-dependent pathway, previous research suggests the existence of an alternative trafficking mechanism for HRas. Furthermore, it seems that this alternative mechanism does not use either the Golgi or vesicles as the primary mode of intracellular trafficking. Based on these earlier findings, in this study we attempted to gain further insights into the Golgi-independent trafficking.

Golgi-independent trafficking exists in multiple cell types

Golgi-dependent and Golgi-independent trafficking mechanisms occur across multiple cell types, including NIH 3T3, Cos-7, MDCK and HeLa. Although the degree of emphasis on the classical trafficking pathway is slightly less in MDCK than other cell types, it does not lessen the observation that the presence of GFP affects protein trafficking. These results imply the general existence of the Golgi-independent mechanism.

Palmitoylation is required for Golgi-independent trafficking mechanism

With the importance of the Golgi independent mechanism already established, this study was expanded toward exploring which properties of HRas might act as contributing factor(s) in selecting trafficking mechanisms in the ER. Because farnesylation, the first step in HRas

modification, occurs in the cytosol after which the proteins are recruited to the ER. Further, because trafficking selection appears to happen in the ER, we postulated that palmitoylation is a key element in HRas trafficking. Our data show that while single palmitoylation at either position 181 or 184 is sufficient to localize HRas to the plasma membrane, palmitoylation of both cysteines is required for HRas to travel via Golgi-independent, non-vesicular exocytosis.

Residues between the lipid moieties are important in HRas trafficking

Our previous data demonstrate that the HRas tail—the final 10 HRas amino acid residues—are sufficient to locate GFP to the plasma membrane. The HRas tail can use both the classical vesicular and the newly identified non-vesicular mechanisms to reach plasma membranes. To explore precisely which residues in the HRas c-terminus participate in trafficking mechanism selection, amino acids placed between the two palmitate groups were mutated from —CMCKCVLS (HRas wt or HRas 61L) to —CDQCECVLS (HRas CDQCE). HRas CDQCE never reached the plasma membrane even when proteins were expressed for up to 48 hours, which indicates that the CAAX box, the lipid modified cysteines, and the surrounding residues are factors in proper HRas localization and trafficking.

Conservation of HRas hypervariable region and Golgi-independent trafficking

The HRas hypervariable region spans from residue 166 to residue 189. In this study, we asked how much of the hypervariable region must be conserved in order for HRas to use a Golgi-independent mechanism to reach the plasma membrane. To answer this question, GFP delta 17 and GFP delta 19 constructs were obtained. In the GFP delta 17 construct, 7 alanines were substituted for residues 173 to 181, and in the GFP delta 19 construct, 7 alanines were substituted for residues 166 to 172. Although both mutants can easily reach the plasma

membrane when unperturbed, they are unable to traffic to the plasma membrane in the presence of either BFA or nocodazole.

These results indicate that conservation of the amino acids in the hypervariable region is important for HRas localization and trafficking. More specifically, at least one modified cysteine, the CAAX box, and amino acids surrounding the palmitoylated cysteines seem to be factors in HRas trafficking to the plasma membrane. This is important because non-lipid-modified amino acids in the hypervariable region have been largely ignored.

Concluding remarks

Results presented here support the idea that HRas can use a Golgi-independent mechanism to reach the plasma membrane. However, this mechanism is slightly problematic for HRas because of its lipid modifications. In the Golgi-independent mechanism, a fully modified HRas must leave the endomembrane and travel across the cytosol in order to reach the plasma membrane, but lipid groups are highly hydrophobic and, as such, are thermodynamically unfavorable for exposure to the aqueous phase of the cytosol.

HRas may overcome this problem in one or more of three possible ways. First, the lipid groups within an HRas molecule may fold inward to be shielded from the cytosol. However, there is currently no evidence that support this model. Alternatively, HRas can travel in small particles called “rasosomes” [20], with a chaperone or an escort protein. An escort or chaperone protein might be able to protect the lipid moieties until HRas reaches the plasma membrane. Finally, it may be possible that HRas proteins can form oligomers with one another. In forming an oligomer, HRas lipid groups might be oriented inward and thereby shielded from the cytosol.

We have shown that conservation of the HRas hypervariable region is important for Golgi-independent trafficking. Further we have shown that the addition of GFP greatly enhances the Golgi-dependent trafficking mechanism. Based on data presented here, the formation of oligomers or the binding of chaperone or escort proteins seem most likely. Although additional studies are required, it is tempting to speculate that the hypervariable region serves as a recognition sequence for chaperone binding and that the GFP tag prevents oligomers or chaperone/escort protein complexes from forming.

Whatever the actual mechanism might be, information presented in this study could be useful in developing future cancer therapies. Currently, treatments for cancer focus on HRas membrane localization, Golgi-mediated trafficking, and mutations in the switch regions. However, further research into the HRas hypervariable region and the inhibition of non-vesicular trafficking could lead to effective cancer treatments.

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Figure Legends

All scale bars represent 10µm. White arrowheads indicate the plasma membrane and blue arrowheads indicate the Golgi unless otherwise noted.

Figure 1 GFP-HRas Q61L and mGFP-HRas Q61L accumulates in the Golgi area

- A) NIH 3T3 cells were transfected with GFP-HRas Q16L (green) for 24 hours then co-immuno-labeled with giantin antibody (red).
- B) NIH 3T3 cells were transfected with mGFP-HRas Q61L (green) for 24 hours then co-immuno-labeled with giantin antibody (red).

Figure 2 GFP-HRas Q61L expression in multiple cell types

- A) NIH 3T3 cells were transfected with GFP-HRas Q16L (green) for 24 hours then co-immuno-labeled with giantin antibody (red).
- B) HeLa cells were transfected with GFP-HRas Q16L (green) for 24 hours then co-immuno-labeled with giantin antibody (red).
- C) MDCK cells were transfected with GFP-HRas Q16L (green) for 24 hours then co-immuno-labeled with giantin antibody (red).

D) Cos-7 cells were transfected with GFP-HRas Q16L (green) for 24 hours then co-immuno-labeled with giantin antibody (red).

Figure 3 GFP-HRas Q61L utilizes Golgi-independent, microtubule-independent exocytosis

All cells were transfected with GFP-HRas Q16L (green), then immediately treated with cycloheximide for 6 hours.

- A) After the cycloheximide treatment, GFP-HRas Q61L-expressing NIH 3T3 cells were incubated in media for 4 hours.
- B) After the cycloheximide treatment, GFP-HRas Q61L-expressing NIH 3T3 cells were incubated in BFA for 4 hours.
- C) After the cycloheximide treatment, GFP-HRas Q61L-expressing NIH 3T3 cells were incubated in nocodazole for 4 hours.
- D) After the cycloheximide treatment, GFP-HRas Q61L-expressing NIH 3T3 cells were incubated in media for 4 hours.
- E) After the cycloheximide treatment, GFP-HRas Q61L-expressing NIH 3T3 cells were incubated in BFA for 4 hours.
- F) After the cycloheximide treatment, GFP-HRas Q61L-expressing NIH 3T3 cells were incubated in nocodazole for 4 hours.

Figure 4 HRas mono-palmitoylated mutants

HRas Q61L contains two palmitoylation sites (blue) at positions 181 and 184 and a farnesylation site at 186 (red). HRas C181S mutants contain one palmitoylation site at position 184 and a farnesylation site at position 186. Cysteine 181 has been mutated from cysteine to serine (orange). For HRas C184S mutant, cysteine at position 184 has been mutated to serine.

Figure 5 Mono-palmitoylated HRas does not utilize Golgi-independent, microtubule-independent exocytosis

- A) YFP-GT46 was expressed in NIH 3T3 cells for 28 hours. The yellow arrowhead shows the Golgi accumulation and the white arrowhead shows the plasma membrane localization.
- B) 18 hours after transfection, YFP-GT46-expressing NIH 3T3 cells were treated with cycloheximide for 6 hours, then the cells were treated with nocodazole for an additional 4 hours before fixation.
- C) 18 hours after transfection, YFP-GT46-expressing NIH 3T3 cells were treated with cycloheximide for 6 hours, then the cells were incubated in BFA for an additional 4 hours before fixation.
- D) HRas C181S NIH 3T3 cells 28 hours after transfection. The white arrowhead shows the plasma membrane localization.
- E) 18 hours after transfection, HRas C181S-expressing NIH 3T3 cells were treated with cycloheximide for 6 hours, then the cells were treated with nocodazole for an additional 4 hours before fixation.
- F) 18 hours after transfection, HRas C181S-expressing NIH 3T3 cells were treated with cycloheximide for 6 hours. Then the cells were treated with BFA for an additional 4 hours before fixation.
- G) HRas C184S-expressing NIH 3T3 cells 28 hours after transfection. The white arrowhead shows the plasma membrane localization
- H) 18 hours after transfection, HRas C184S-expressing NIH 3T3 cells were treated with the cycloheximide for 6 hours, then the cells were treated with nocodazole for an additional 4 hours before fixation.

- I) 18 hours after transfection, HRas C184S-expressing NIH 3T3 cells were treated with cycloheximide for 6 hours, then the cells were treated with BFA for an additional 4 hours before fixation.

Figure 6 Mono-palmitoylated HRas does not retreat back into ER in the presence of BFA

- A) NIH 3T3 cells were transfected with HRas C181S mutant for 18 hours, then treated with BFA for additional 3.5 hours. Cells were co-immuno-labeled for HRas (green) and trap (red).
- B) Merged image of HRas C181S (green) and ER (red).
- C) NIH 3T3 cells were transfected with HRas C184S mutant for 18 hours, then were treated with BFA for an additional 4 hours. Cells were co-immuno-labeled for HRas (green) and trap (red).
- D) Merged image of HRas (green) C184S and trap (red).

Figure 7 HRas CDQCE mutant does not localize to the plasma membrane

- A) NIH 3T3 cells were transfected with HRas Q61L for 24 hours then immuo-labeled. The red arrowhead indicates the Golgi and the white arrowhead indicates the plasma membrane.
- B) NIH 3T3 cells were transfected with HRas CDQCE WT for 24 hours then immuo-labeled.

Figure 8 HRas alanine mutants

HRas Q61L contains two palmitoylation sites (blue) at positions 181 and 184 and a farnesylation site at 186 (red). The HRas delta 17 mutant contains all of the lipid modification sites but has 7 alanine substitutions (orange) far upstream from the

CAAX box. HRas delta 19 also has all of the lipid modification sites but its alanine substitutions (orange) are immediately upstream of the CAAX box.

Figure 9 HRas alanine mutants do not utilize the Golgi-independent, microtubule-independent exocytosis mechanism.

- A) HRas delta 17 in NIH 3T3 cells 24 hours after transfection.
- B) HRas delta 19 in NIH 3T3 cells 24 hours after transfection.
- C) NIH 3T3 cells were transfected with HRas delta 17 mutant, then immediately treated with the cyclohexoimide for 6 hours. The cells were then treated with BFA for an additional 4 hours before fixation.
- D) NIH 3T3 cells were transfected with HRas delta 19 mutant, then immediately treated with the cyclohexoimide for 6 hours. The cells were then treated with BFA for additional 4 hours before fixation.
- E) NIH 3T3 cells were transfected with HRas delta 17 mutant, then immediately treated with the cyclohexoimide for 6 hours. The cells were then treated with nocodazole for an additional 4 hours before fixation.
- E) NIH 3T3 cells were transfected with HRas delta 19 mutant, then immediately treated with the cyclohexoimide for 6 hours. The cells were then treated with nocodazole for an additional 4 hours before fixation.

Table Legends

Table 1 HRas perinuclear distribution is distinct from the pattern of a protein in the classical vesicular trafficking pathway in NIH 3T3 and Cos-7 cells

NIH 3T3 cells and Cos-7 cells were co-transfected with YFP-GT46 and one of the H-Ras DNAs was indicated. After 18 hours, the cells were fixed and immuno-stained for HRas. Approximately 100 cells were examined and the strength of the HRas signal in the perinuclear area was assigned values of “none”, “+”, or “++”. Values are the

averages and standard deviations of the number of cells from two (HRas) or four (YFP-GT46) independent experiments. Monomeric GFP is abbreviated as mGFP and N.d. means not determined.

Table 2 HRas perinuclear distribution is distinct from the pattern of a protein in the classical vesicular trafficking pathway in MDCK and HeLa cells

MDCK cells, and HeLa cells were co-transfected with YFP-GT46 and one of the H-Ras DNAs was indicated. After 18 hours, the cells were fixed and immuno-stained for HRas. Approximately 100 cells were examined and the strength of the HRas signal in the perinuclear area was assigned values of “none”, “+”, or “++”. Values are the averages and standard deviations of the number of cells from two (HRas) or four (YFP-GT46) independent experiments. Monomeric GFP is abbreviated as mGFP and “N.d.” means not determined.

Figures

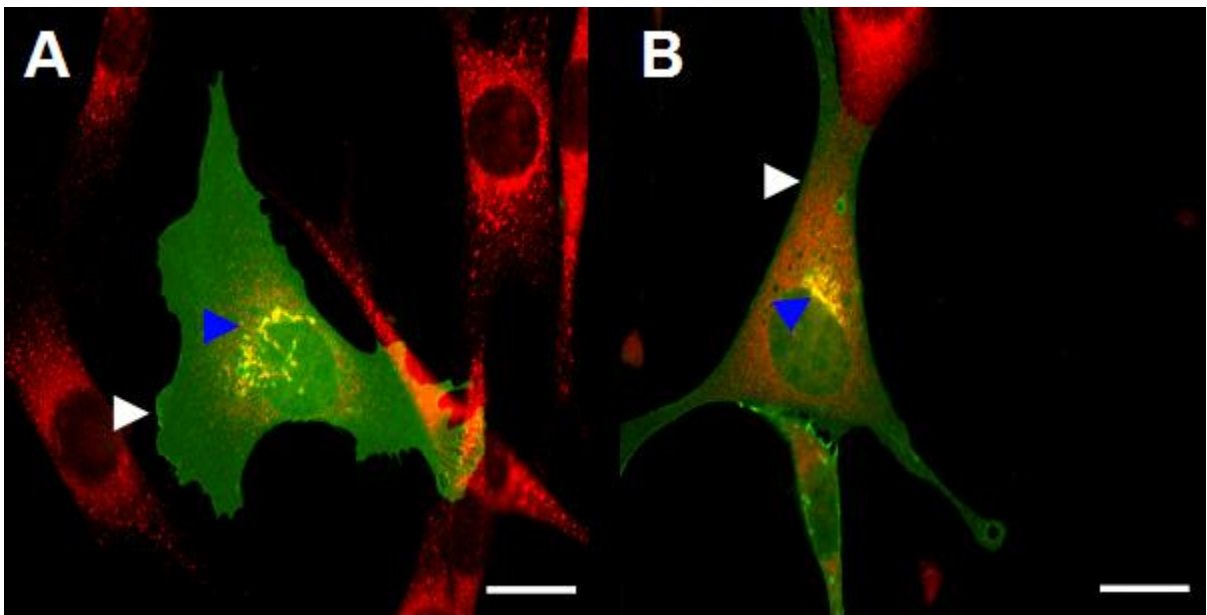


Figure 1 GFP-HRas Q61L and mGFP-HRas Q61L accumulate in the Golgi area

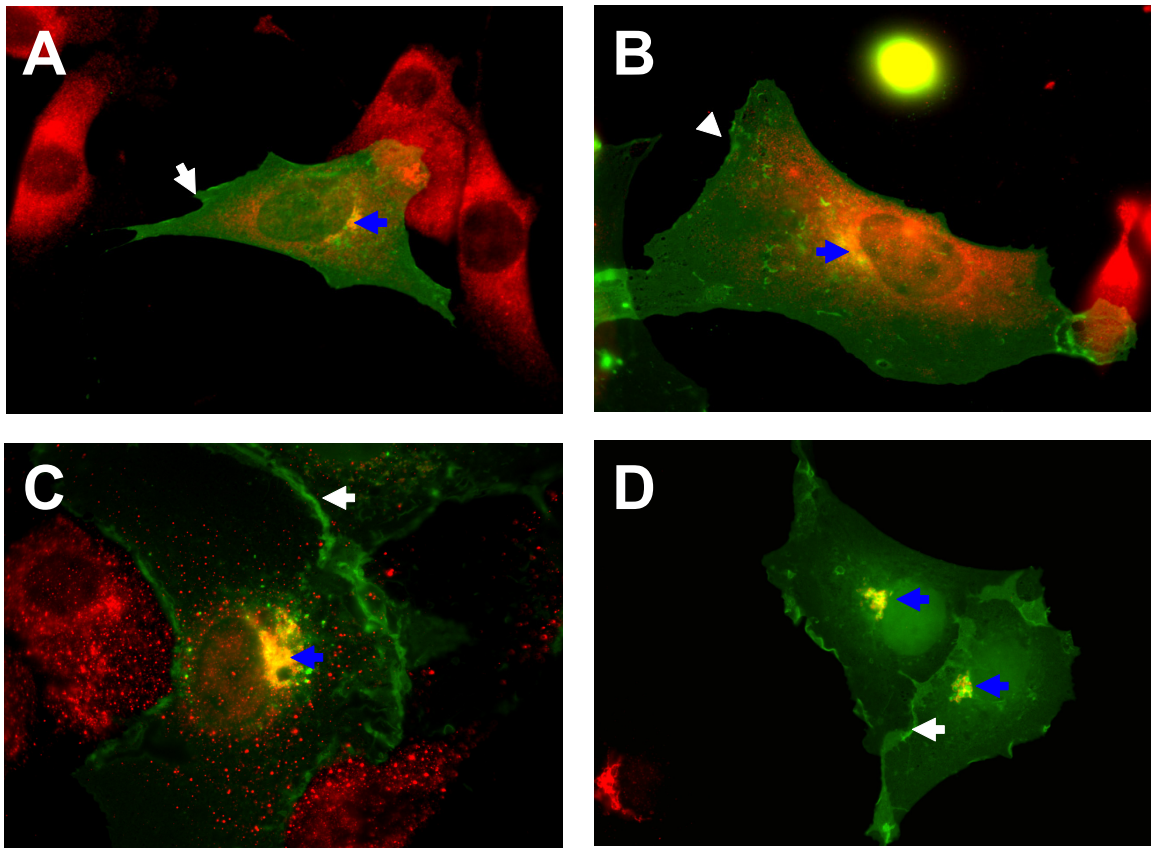


Figure 2 GFP-HRas Q61L expression in multiple cell types

Table 1 HRas perinuclear distribution is distinct from the pattern of a protein in the classical vesicular trafficking pathway in NIH 3T3 and Cos-7 cells.

		Cell Type					
		NIH-3T3			COS-7		
Fluorescence in perinuclear area		++	+	None	++	+	None
D N A T Y P E	YFP-GT46	92 ± 9	4 ± 3	5 ± 5	94 ± 3	2 ± 1	3 ± 2
	H-Ras ^{wt}	6 ± 5	10 ± 6	83 ± 1	4 ± 1	3 ± 2	93 ± 3
	H-Ras ^{Q61L}	1 ± 1	5 ± 4	94 ± 4	7 ± 4	9 ± 4	83 ± 8
	GFP-H-Ras ^{wt}	45 ± 14	23 ± 2	32 ± 15	50 ± 2	17 ± 3	33 ± 1
	GFP-H-Ras ^{Q61L}	59 ± 5	6 ± 8	36 ± 3	59 ± 12	14 ± 4	28 ± 8
	mGFP-H-Ras ^{Q61L}	23 ± 2	23 ± 2	51 ± 5	n.d.	n.d.	n.d.

Table 2 HRas perinuclear distribution is distinct from the pattern of a protein in the classical vesicular trafficking pathway in MDCK and HeLa cells.

		Cell Type					
		MDCK			HeLa		
Fluorescence in perinuclear area		++	+	None	++	+	None
	YFP-GT46	45 ± 12	26 ± 12	30 ± 0	90 ± 4	1 ± 2	9 ± 3
	H-Ras ^w	5 ± 1	4 ± 2	91 ± 3	5 ± 1	4 ± 2	88 ± 3
	H-Ras ^{Q61L}	1 ± 1	1 ± 1	98 ± 0	7 ± 2	2 ± 1	91 ± 1
	GFP-H-Ras ^w	14 ± 1	10 ± 7	77 ± 8	72 ± 4	7 ± 3	22 ± 5
	GFP-H-Ras ^{Q61L}	35 ± 4	18 ± 5	54 ± 8	45 ± 8	7 ± 1	48 ± 7
	mGFP-H-Ras ^{Q61L}	n.d	n.d	n.d	n.d	n.d	n.d

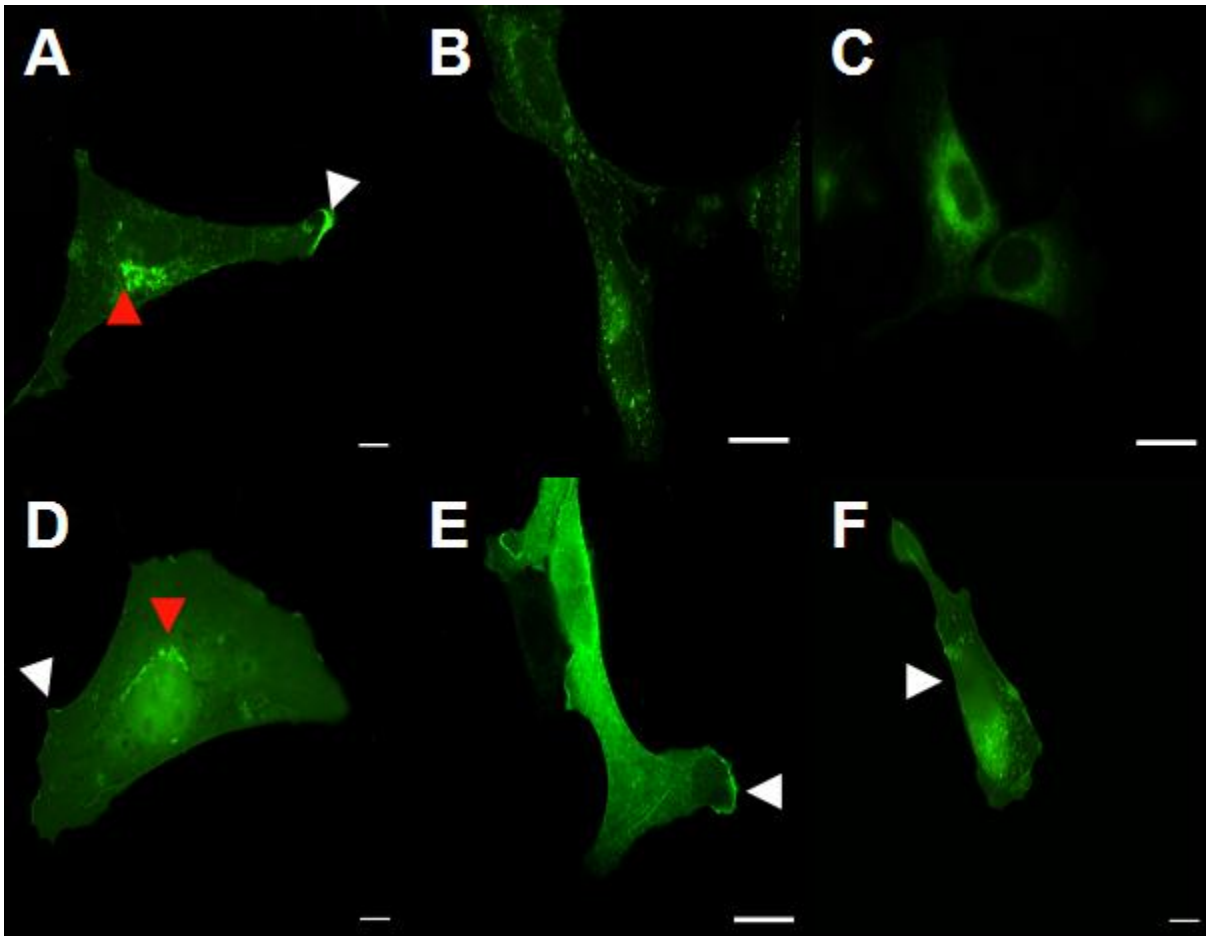


Figure 3 GFP-HRas Q61L utilizes Golgi-independent, microtubule-independent exocytosis

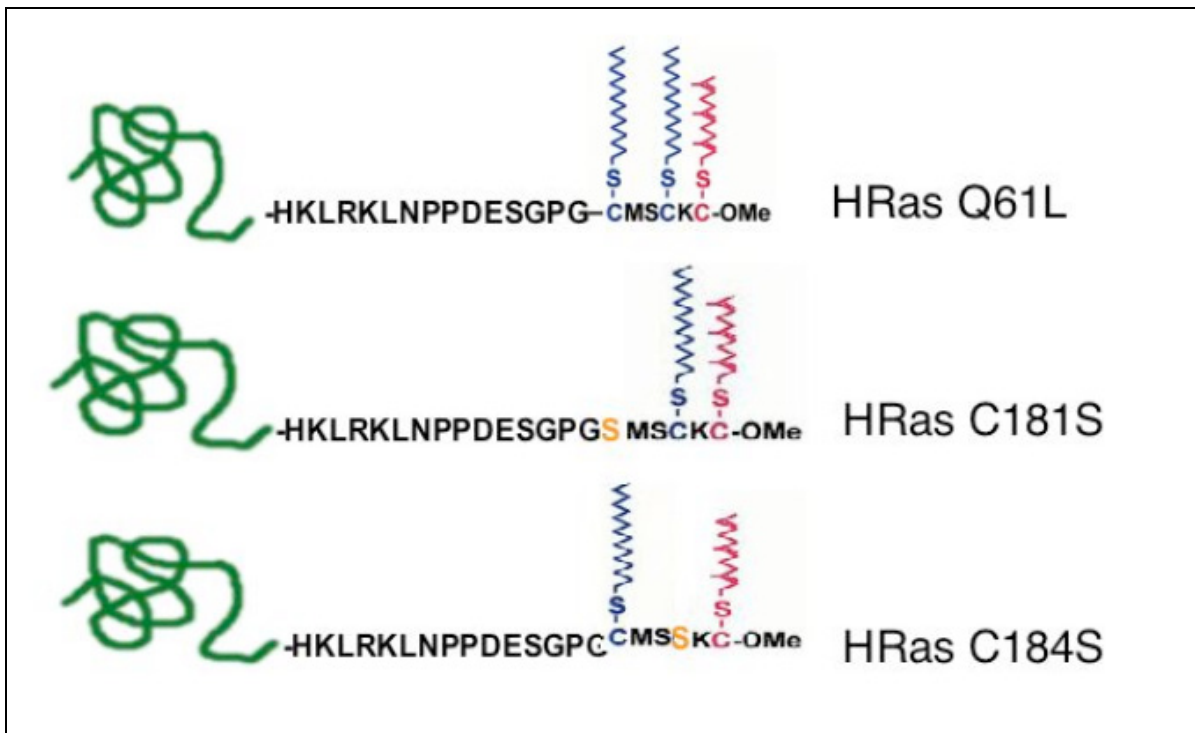


Figure 4 HRas mono-palmitoylated mutants

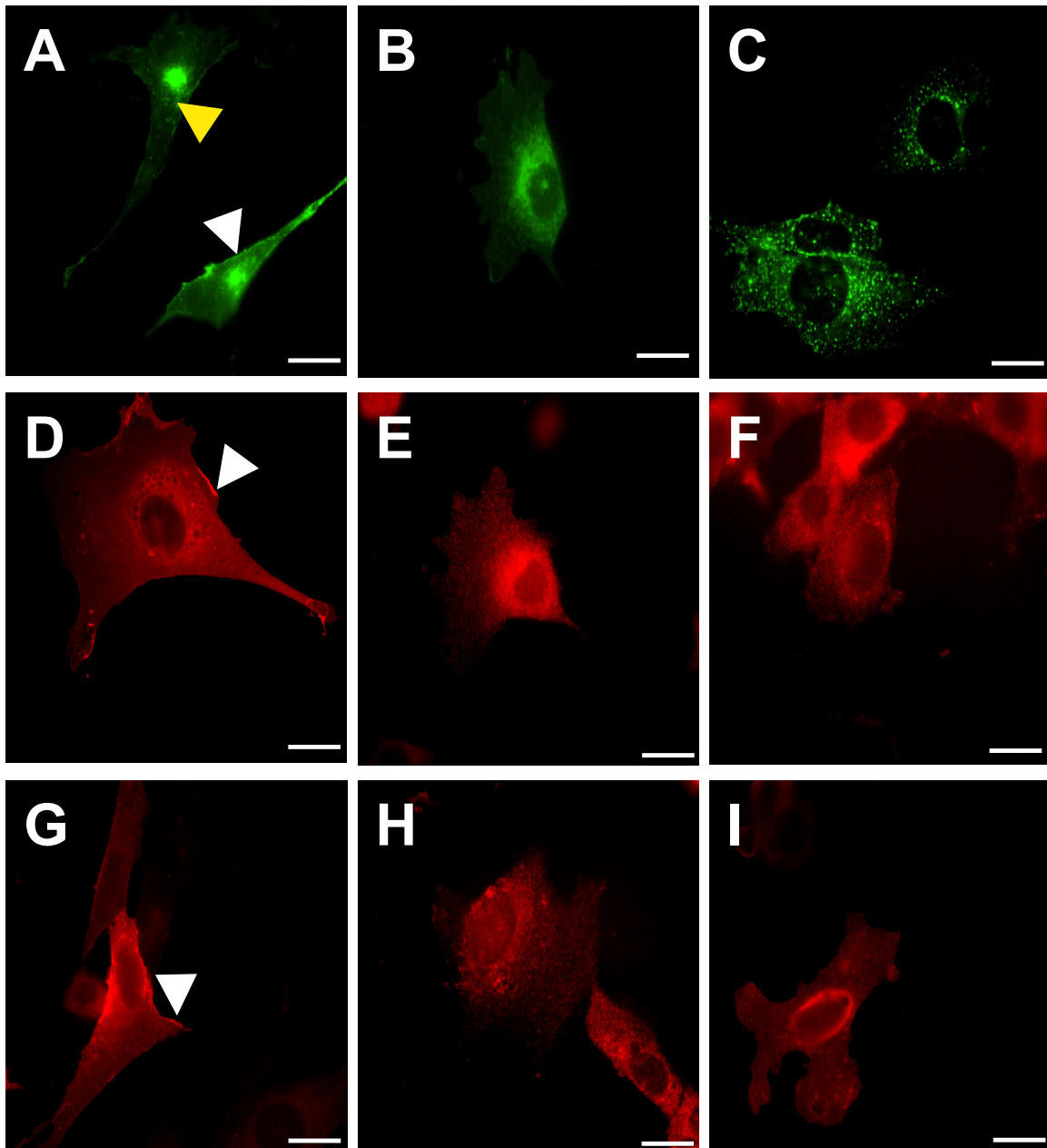


Figure 5 Mono-palmitoylated HRas does not utilize Golgi-independent, microtubule-independent exocytosis

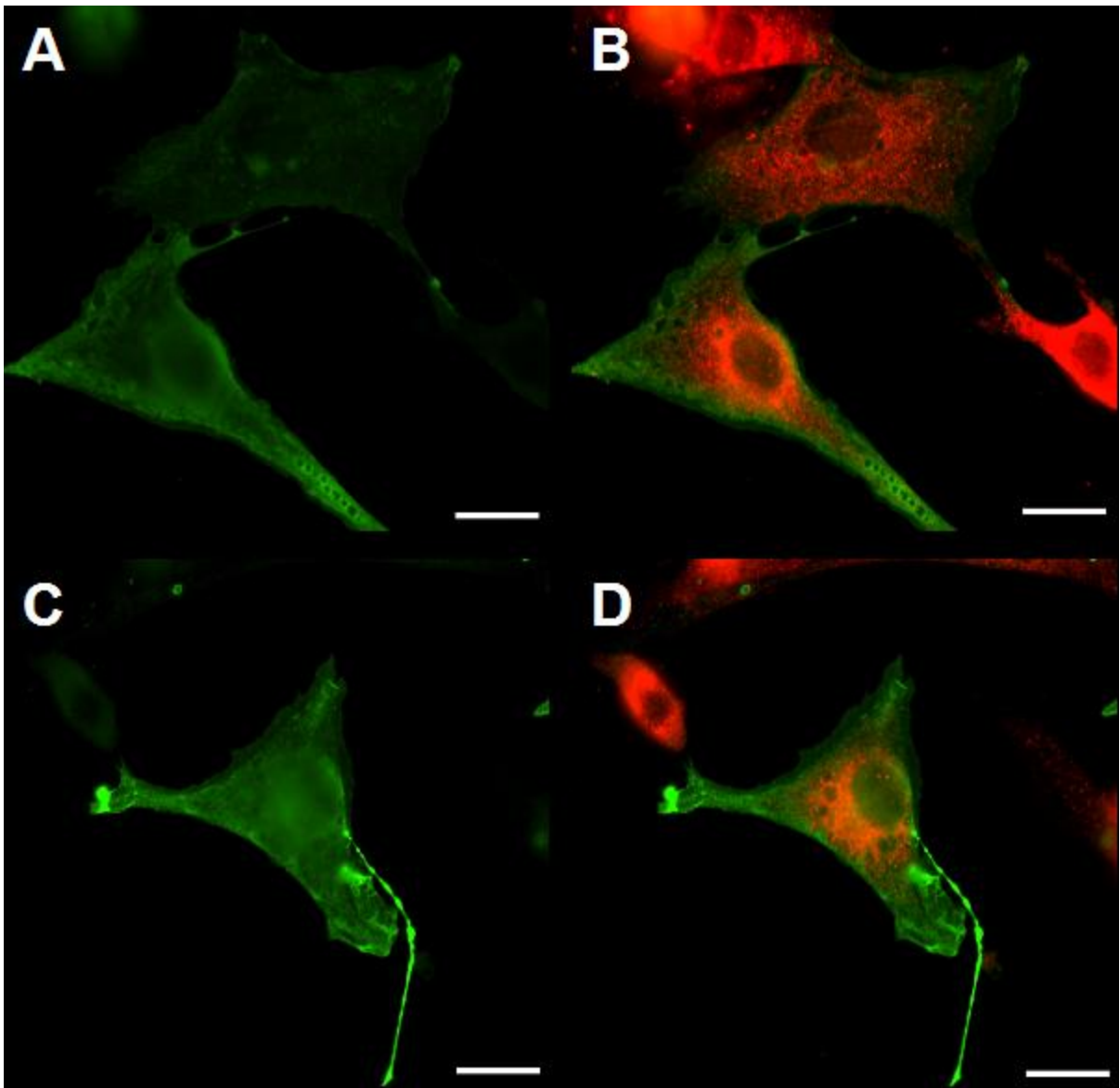


Figure 6 Mono-palmitoylated HRas does not retreat back into ER in the presence of BFA

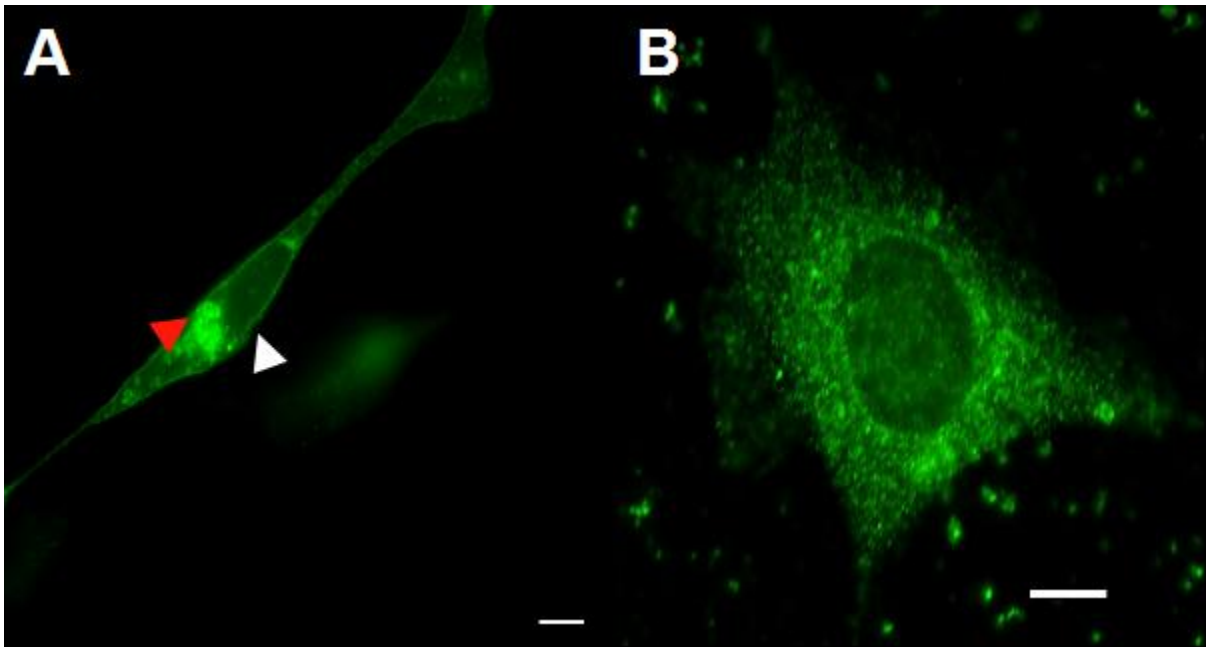


Figure 7 HRas CDQCE mutant does not localize to the plasma membrane

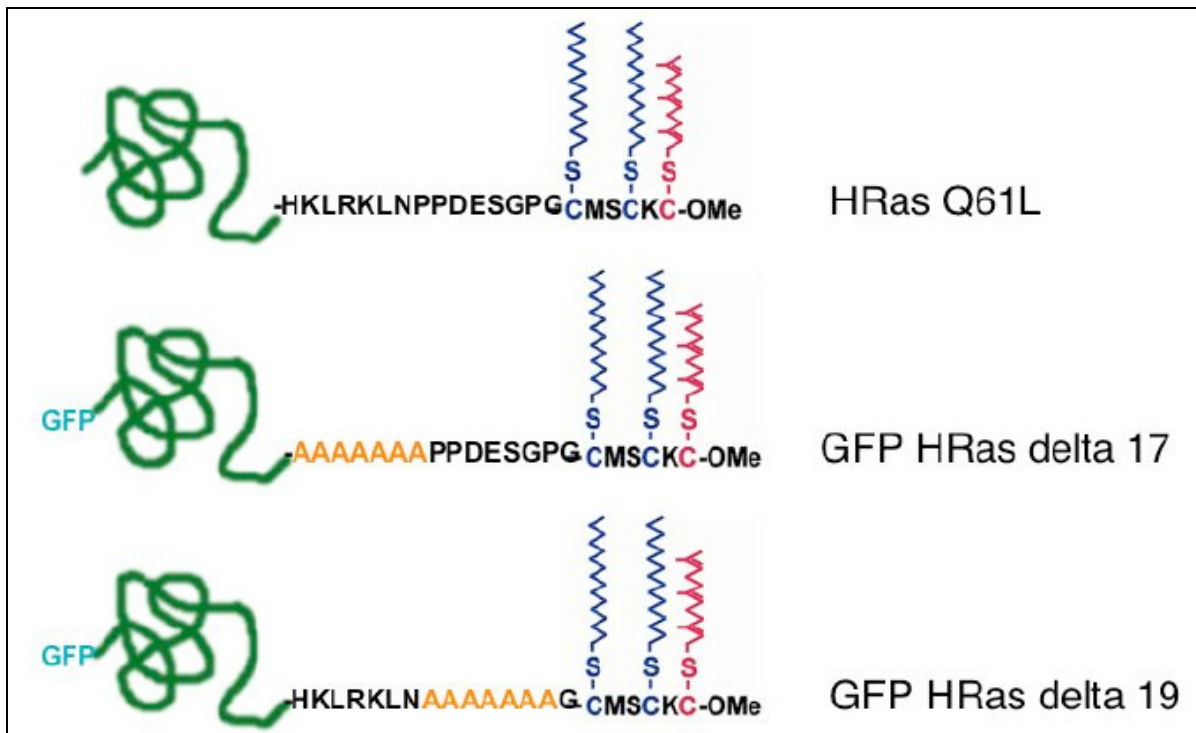


Figure 8 HRas alanine mutants

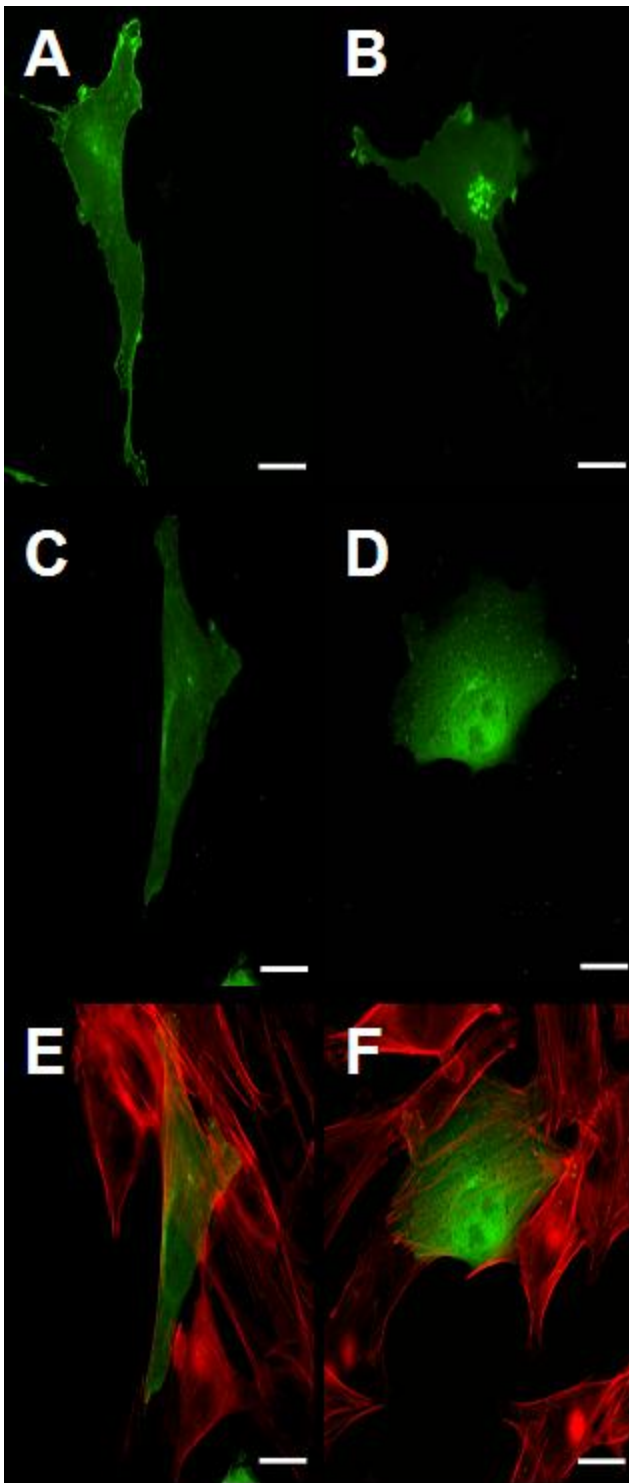


Figure 9 HRas alanine mutants do not utilize the Golgi-independent, microtubule-independent exocytosis mechanism

Both active and inactive forms of H-Ras bind to clathrin-independent endosomes associated with ARF6

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Running title: H-Ras binds Arf6-associated vesicles

Key words: protein trafficking, perinuclear recycling center, endocytosis, clathrin

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Abstract

H-Ras has often been sighted on endocytic vesicles, and, recently, on recycling endosomes, but the class of endosomes to which it bound has remained uncharacterized. The aim of this study was to identify those pathway(s), and to determine if endosomal H-Ras could bind GTP or was inactive. The results found that ectopically expressed, constitutively active H-Ras61L or normal H-Ras, in either basal or EGF-stimulated NIH 3T3 cells, were absent from endosomes of the clathrin-associated pathway. Instead, both forms of H-Ras were present on endosomes of the clathrin-independent, Arf6-associated pathway and in the endosomal-recycling center. In contrast, the Ras effector, Raf-1, was distributed primarily on EEA1-containing endosomes of the clathrin-dependent path. K-Ras4B-V12 and chimeric green fluorescent proteins with either H-Ras or K-Ras4B C-termini were also present on Arf6-associated vesicles. These results identify a specific class of constitutive endosomal vesicles

that can bind Ras proteins. This information provides a new mechanism through which membrane-bound H-Ras can localize on intracellular membranes.

Introduction

Although the site of Ras signaling is widely depicted as the plasma membrane, Ras proteins have now been shown to be distributed on, and to produce significant signals from, internal membranes [1-3]. The means by which Ras proteins localize to these internal sites are varied. In neurons, calcium signals trigger Ca^{++} /calmodulin binding to KRas4B and cause the release of the KRas/calmodulin complex from the plasma membrane [4]. This cytosolic complex is subsequently targeted to Golgi and recycling endosomes [5]. For H-Ras, some of the newly synthesized protein is delivered from the ER to Golgi membranes, apparently by non-vesicular means, and eventually boards outward-bound vesicles within the cytoplasm [6, 7]. Recent work indicates that if palmitoylated H-Ras that is already at the plasma membrane becomes de-palmitoylated, it is released into the cytosol, and can be captured on internal membranes, from which it can then re-join the biosynthetic pathway [8]. This deacylation-controlled internalization appears to be non-vesicular. However, H-Ras has repeatedly been found on vesicles with all the characteristics of endosomes [9-11], including new observations of H-Ras on membranes of the pericentriolar endocytic recycling compartment [12]. A small portion of H-Ras can also become ubiquitinated, a modification that stabilizes its association with endosomes but seems to decrease its association with Golgi membranes [13]. Thus it is unclear whether H-Ras that reaches the plasma membrane must await depalmitoylation before it can re-enter the cell and bind to internal membranes or whether a membrane-bound form of H-Ras can traverse the cytosol and serve as a source for intracellular signaling.

A clear identity for the endosomes to which H-Ras binds has not been reported. There is even the possibility that more than one type of endosomal pathway might be utilized, as the distribution of H-Ras in the plasma membrane is reported to vary. The inactive form of H-Ras is included in raft-like lipid microdomains, while GTP-bound forms inhabit the more general lipid bilayer [14]. Endocytic pathways also show preferences for one type of domain or the other [15]. A previous study has shown that a Rab5- and dynamin-regulated pathway of endocytosis and recycling is required for signaling by the Ras effector, Raf-1 [16]. Additional studies report that both Rab5 and Rab11 regulate H-Ras internalization [12]. However, Rab5 is found on endosomal membranes and sorting endosomes that are derived from both clathrin-mediated and clathrin-independent pathways [12, 17], and thus does not define a specific class of endosomes. Expression of the constitutively active Rab5-Q79L protein causes some H-Ras to accumulate on enlarged endosomes, but, notably, not Raf-1 [18]. Thus, a portion of H-Ras can be internalized, but the internalized population appears to disengage from this effector. Raf-1 itself has also been observed on endosomes, colocalizing with EEA1, an early endosomal marker for the clathrin-mediated pathway [10]. Other studies report that blocking clathrin-mediated endocytosis with a dominant, mutant form of epsin or AP180 does not prevent accumulation of H-Ras on intracellular membranes [2, 8]. In addition, in earlier electron microscopy studies, H-Ras was notably absent from clathrin-coated pits or flat arrays on the plasma membrane [14, 19, 20]. Although these studies suggest that H-Ras can leave the plasma membrane and enter an endocytic pathway, they do not provide a clear identity for that pathway, or answer whether activated H-Ras can maintain its GTP-bound state while on such endosomes.

Several pathways for clathrin-independent endosomal entry of proteins have been described [15, 17, 21-24]. One of these is a pathway that serves as an entry route for the class I major histocompatibility complex protein (MHC-I), IL-2 receptor α subunit, the β -1 subunit of integrins, and the Src tyrosine kinase [25-27]. This pathway is identifiable by the presence of Arf6. Arf6 is a member of the Arf and Arl subfamily of small GTPases that participate in vesicular trafficking [28]. The Arf6 protein is located on the plasma membrane, endosomes, and tubular-vesicular recycling membranes [29]. Several studies suggest that Arf6-associated vesicles converge on the endosomal recycling center (ERC) [30], where they mingle with endosomes that are derived independently from clathrin-mediated endocytosis [26, 31]. Arf6-associated vesicles also participate in a recycling pathway that returns proteins to the plasma membrane [32, 33].

The studies reported here examined what type of endosomes could bind H-Ras, whether binding differed between inactive or active forms, and if activation changed the type of endosome to which H-Ras was bound. The results build a new mechanistic link between Arf6 and H-Ras that reaches from the plasma membrane to the perinuclear endosomal recycling center.

Materials and methods

Plasmids, cell culture, and DNA transfection

The H-Ras and ExtRas DNAs in the pcDNA3 vector were described previously [34]. GFP-H-Ras-61Q, GFP-KRas4B-G12V, GFP-ExtRas61L, GFP-ExtRas-61Q, and GFP-H-Ras-61L were constructed using the pcDNA3 versions as PCR templates and transferred to the pEGFP-C3 vector (Clontech Labs). GFP-RBD contains residues 51-131 of Raf-1 [2] that were transferred to pEGFP-C3 from a cDNA for a GST-RBD that was obtained from the

laboratory of C. Der (UNC). GFP-H-tail was constructed by attaching the bases encoding the 10 C-terminal amino acids of H-Ras (GCMCKCVLS), a termination codon, and a Bam HI restriction enzyme site to pEGFP-C3 by PCR. Arf6-67Q-GFP (Arf6-WT, wild type) and Arf6-Q67L-GFP are in the pEGFP-N1 vector; Arf6-67Q-HA and Arf6-Q67L-HA each with a C-terminal hemagglutinin (HA) epitope tag, are in pXS vectors. Dr. J.G. Donaldson, National Institute of Health generously provided all the Arf6 constructs and the GFP-K-tail plasmid.

NIH 3T3 cells were cultured at 37°C and 10% CO₂ in Dulbecco's modified Eagle's medium supplemented with 10% calf serum plus penicillin, streptomycin, glutamine and sodium pyruvate. For immunofluorescence and immuno-isolation, NIH 3T3 cells were transfected with Effectene (Qiagen) according to the manufacturer's instructions and used after 24 hours or as mentioned in the results. The H-Ras61L or H-RasWT (HRas cellular form, wild-type) stable cell lines have the H-Ras61L or H-RasWT DNAs expressed from the pZIPneo vector; the GFP-H-Ras61L stable cell line has the GFP-H-Ras61L DNA expressed from the pEGFP-C3 vector. Cells treated with EGF (Gibco BRL) were cultured in the same media described above and incubated with 5 or 10 ng/ml of EGF at 37°C for the time as indicated, followed by washing with phosphate-buffered saline and fixation.

Antibodies

Antibodies used for immunoblotting included mouse monoclonal anti-Ras (BD Bioscience 610010), mouse monoclonal anti-c-Raf (BD Bioscience 61051), rabbit polyclonal anti-hemagglutinin (Covance PRB-101P), mouse monoclonal anti-GFP (Roche 1814460) and rabbit polyclonal anti-EEA1 (ABR P1-063). For immunofluorescence, rat monoclonal anti-Ras (sc-34), rabbit polyclonal anti-c-Raf-1 (sc-227), and rabbit polyclonal anti-clathrin heavy

chain (sc-9069) were from Santa Cruz Biotechnology. Mouse monoclonal antibody (W6/32) to human MHC-I protein was from Leinco Technologies (#H263). The same EEA1 antibody or mouse anti-hemagglutinin (Covance PRB-101R) were used for both immuno-isolation and immunofluorescence. Murine monoclonal anti-Ras (Quality Biotech LA069) and the above rat monoclonal anti-Ras antibody are also used for immuno-isolation. Goat anti-rat IgG coupled to Alexa Fluor 488, goat anti-mouse IgG coupled to Alexa Fluor 594, goat anti-rabbit IgG coupled to Alexa Fluor 594, donkey anti-rat IgG coupled to Alexa Fluor 594, and transferrin from human serum coupled to Alexa Fluor 594 are from Molecular Probes.

Live cell labeling with transferrin or MHC-I antibody

To mark early endosomes of the clathrin-dependent pathway, cells were transfected and incubated for 18 hours, then cycloheximide (10 $\mu\text{g/ml}$) was added for 5.25 hours, to allow time for previously synthesized protein to vacate Golgi membranes. Cells were then chilled for 45 minutes at 4°C in a cold room in medium containing 50 mM HEPES, pH 7.4 buffer, cycloheximide (10 $\mu\text{g/ml}$), and AlexaFluor-transferrin (Molecular Probes, 10 $\mu\text{g/ml}$). Finally, the cold medium was removed and pre-warmed (37°C) medium containing 50 mM HEPES, pH 7.4 was added and cells were incubated at 37°C in a water bath for 1 minute before fixation with cold 4% paraformaldehyde and processing for immunofluorescence.

For marking the endosomal recycling center (ERC), cells were transfected with various cDNAs, incubated for 18 hours, and then treated for 5.25 hours with cycloheximide (10 $\mu\text{g/ml}$). Alexa-Fluor transferrin (10 $\mu\text{g/ml}$) was then added for 45 minutes. Cells were washed, fixed and processed for immunofluorescence.

To monitor MHC-I internalization, HeLa cells were transfected and incubated for 18 hours, then cycloheximide (10 $\mu\text{g/ml}$) was added for 5.5 hours, to allow time for newly

synthesized H-Ras proteins to vacate Golgi membranes. Cycloheximide-containing media with MHC-I antibody (200µg/ml) was equilibrated to 37° C and then added to the above cells for 30 min at 37° C. At the end of incubation, cell surface-associated antibodies were removed by rinsing the cells in low pH solution (0.5% acetic acid, 0.5M NaCl, pH 3.0) for 30 seconds [26]. Cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) for 10 minutes, permeabilized, and stained in blocking buffer (2% horse serum, 0.4% bovine serum albumin and 0.2% saponin in PBS, pH 7.4) [26, 35]. The internalized antibodies were visualized with the appropriate fluorochrome-labeled secondary antibody in the above blocking buffer.

Immuno-isolation of membranes

Twenty-four hours after transfection, cells were scraped into 300 µl homogenization buffer (3 mM imidazole, pH 7.4, 1 mM Na₃VO₄, 5 mM MgCl₂, 1% Protease Inhibitor Cocktail (Sigma), and 5% sucrose) and homogenized with 15 strokes in a Dounce homogenizer followed by 20 passages through a 26-gauge needle. The nuclei and unbroken cells were removed by centrifugation at 1300 x g (4 °C) for 10 min. This post-nuclear supernatant (PNS) was pre-cleared by incubation for 1 hour at 4°C with protein G- or protein A-agarose beads (Invitrogen). Beads were removed and the supernatant then incubated with mouse monoclonal anti-HA antibody or a mixture of mouse and rat monoclonal anti-H-Ras antibodies or rabbit EEA1 antibody overnight. The following day fresh protein G agarose beads (or protein A beads for rabbit EEA1 antibody) were added for 2 hours at 4°C. The beads were washed 3 times in cold homogenization buffer and boiled in SDS-PAGE sample buffer. Proteins present in the membranes were separated by SDS-PAGE and detected on

immunoblots with the indicated antibodies. 95% of the sample was loaded in the IP lane, and 5% in the PNS lane.

Immunoblotting

Samples collected by immuno-isolation were separated by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Pall Life Science) and then incubated in blocking buffer (Vector Labs 10x casein diluted to 1x) in TTBS [0.15 mol/L NaCl, 0.05 mol/L Tris-HCl, 0.05% Tween-20, pH 7.4] for 20 minutes at room temperature. PVDF membranes were washed in TTBS, incubated with primary antibodies overnight at 4°C. The proteins of interest were detected using horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies and an enhanced chemiluminescence kit from Pierce.

Immunofluorescence and preparation of sonicated membrane fragments

NIH 3T3 cells were plated on coverslips coated with 100 µg/ml poly-L-lysine (Sigma) and 500 µg/ml fibronectin (Sigma) and transfected with DNA. After 24 hours (or as mentioned in the results), cells were fixed with fresh 4% formaldehyde in phosphate-buffered saline, pH 7.4, at room temperature for 1 hour then permeabilized with -20°C methanol and quenched with 50 mM ammonium chloride in phosphate-buffered saline. The fixed cells were incubated with blocking buffer (2% horse serum and 0.4% bovine serum albumin in phosphate-buffered saline, pH 7.4) then sequentially with primary and secondary antibodies diluted in blocking buffer. Experiments to produce adherent plasma membrane fragments were carried out exactly as described previously [36]. The slips were mounted in Vectashield mounting medium (Vector Laboratories). For experiments imaging adherent membrane fragments, cells were analyzed using a 63X oil immersion lens on a Nikon Eclipse E800

microscope with Spot RT digital camera. All the confocal images were taken with a Leica TCS-NT confocal system with a 63X oil immersion lens with an aperture adjustable from 1.32 to 0.6, using an argon laser (488 nm excitation) and a krypton laser (568 nm excitation). Other images were taken with Leica DMIRE2 microscope using a 100X oil immersion lens with an aperture adjustable from 1.3 to 0.6 and Q Imaging Retiga 1300 camera. Images were exported to Adobe Photoshop 7.0 or, for the images taken with the Leica DMIRE2 microscope; images were taken in black and white, processed in Openlab3.5.1, displayed in colors and then exported to Adobe Photoshop 7.0 to equalize image intensity prior to merging. For some experiments, as indicated in the figure legends, a series of z-stack images were acquired using a motorized stage on the Leica DMIRE2 microscope. These images were processed using Openlab's nearest neighbor deconvolution module and processed as above.

Results

H-Ras does not utilize the clathrin-dependent pathway

The previous report of dynamin sensitivity of H-Ras triggered Raf-1 signaling [16] suggested that the dynamin-sensitive, clathrin-dependent pathway might mediate H-Ras endocytosis. To specifically inspect plasma membranes for possible H-Ras and clathrin colocalization, adherent basal plasma membranes of transfected NIH 3T3 cells were prepared by sonication, fixed, and examined by indirect immunofluorescence [36]. However, neither the activated H-Ras61L (Fig. 1A) nor the transfected cellular form of H-Ras (H-Ras-WT; data not shown) overlapped with clathrin heavy chain.

Another marker of a later step in the clathrin-dependent pathway, the early endosome antigen-1 protein (EEA1), was used to determine if H-Ras was present in early endosomal

vesicles that had already lost their clathrin coat. Upon examination of EEA1-positive vesicles in intact cells, no overlap of H-Ras-WT and EEA1 was detected (Fig. 1B). On adherent fragments of basal membranes from sonicated cells, the population of EEA1-positive vesicles was more clearly resolved, but again no H-Ras (this time the activated form, H-Ras61L) was present in these vesicles (Fig. 1C). Because EEA1 marks early endosomal vesicles specifically, these results indicated that neither an oncogenic nor the normal form of H-Ras were present on early endosomes derived from the clathrin-dependent pathway.

Because the experiments with H-Ras61L used a permanently activated, mutant form of H-Ras, we also tested if H-Ras-WT that became transiently GTP-bound after EGF stimulation now joined the clathrin-dependent pathway. This was a relevant question, as the serum-activated form of H-Ras is reported to shift from raft-like lipid microdomains, to a more general membrane distribution from which clathrin-mediated internalization might occur [14]. However, after stimulation of cells for 10 minutes with a moderate, 10 ng/ml, dose of EGF [37], no co-alignment of the EEA1 marker of early endosomes and H-Ras-WT was detected (Fig. 1D).

As an additional test, a fluorescent derivative of transferrin was added to live cells to enable tracking of the first minutes of clathrin-mediated endocytosis. For these experiments, a cycloheximide pre-treatment was included to avoid imaging newly synthesized H-Ras on Golgi-derived vesicles that might mingle with the endocytic vesicles. Cells were then chilled and incubated with transferrin, so as to label, but prevent internalization of transferrin receptors. Cells were warmed briefly (1 minute) to allow internalization to re-start. Although H-Ras-positive vesicles were present in the re-warmed cells, the transferrin-containing early endosomes did not overlap with GFP-H-Ras61L (Fig. 1E). Thus, although H-Ras was present

on vesicles, it was notably absent from endosomes associated with these characteristic steps of the clathrin-dependent endocytic route.

To supplement the previous imaging results, we used a biochemical approach to examine if H-Ras in its GTP-bound form might be present on EEA1-containing endosomes. Using an antibody to EEA1 and a detergent-free protocol for immuno-isolation of membrane fragments and vesicles [10], the presence of H-Ras and endogenous Raf-1 on EEA1-containing endosomes was assessed. In agreement with the imaging results, neither cellular H-Ras, nor activated H-Ras61L (Fig. 1F) could be detected on the captured EEA1-containing membranes.

Thus, by multiple techniques, we found no evidence of H-Ras on endocytic vesicles that were derived from the clathrin-dependent pathway.

In spite of this absence, endogenous Raf-1 was easily observed in these EEA1 antibody-captured endosomes (Fig. 1F). This ability to capture a protein associated with EEA1-containing membranes verified that the membrane isolation protocol was successful, and thus validated the absence of H-Ras on those membranes. This result confirmed previous work that Raf-1 could be found on EEA1-containing early endosomes [10]. All these studies indicated that endocytic vesicles to which H-Ras was bound had not entered the cells via a clathrin-mediated pathway.

Endosomes associated with Arf6 bind H-Ras

A prominent class of non-clathrin plasma membrane structures is comprised of caveolae. However, the ability of caveolae to internalize (as “caveosomes”) is generally thought to be restricted to special situations such as viral infection [38]. H-Ras, although present on membranes with a low buoyant density similar to that of caveolae, is largely absent from

these structures [14, 36]. Thus another class of non-caveolar, clathrin-independent endosomes for binding H-Ras was sought.

An additional type of clathrin-independent endosomes are those which arise from the constitutive pathway by which the endogenous major histocompatibility complex I (MHC-I) is internalized [30]. To examine this pathway, endosomal vesicles containing MHC-I were identified by applying an antibody that recognizes an external epitope of human MHC-I to live HeLa cells. In addition, to further diminish the signal from endogenous MHC-I and transfected H-Ras that were within the biosynthetic pathway, cycloheximide was added to the transfected cells for the final 6 hours before fixation. Anti-MHC-I antibody was added to the culture medium for the last 30 minutes to label MHC-I at the cell surface. Cells were then briefly washed with mild acid, to remove MHC-I antibody that had not entered the cell, were fixed, and then stained with antibody for the transfected H-Ras protein [35, 39]. The resulting images showed multiple vesicular structures that held both H-Ras and endogenous MHC-I protein, as well as other vesicles that held only a single protein (Fig. 2A).

MHC-I is reported to traffic through a pathway that is associated with the Arf6 small GTPase [40, 41]. The extent of colocalization of externally labeled MHC-I with an expressed Arf6-Q67L tagged with GFP was therefore examined. Similar to the study with H-Ras and MHC-I, there were multiple vesicles that contained both MHC-I and Arf6, and others that held only a single protein (Fig. 2B). These results implied that H-Ras should be present on vesicles on which Arf6 was located. To test this prediction, NIH 3T3 cells were co-transfected with H-Ras-WT and a GFP-tagged version of the cellular form of Arf6 (Arf-WT-GFP), and were examined by fluorescence microscopy. H-Ras-WT was observed on the plasma membrane, as expected, and also on both small endosomes and larger, apparently

fluid-filled, pinosomes that were also populated by Arf6-WT-GFP (Fig. 2C). Pinosome structures are often associated with Arf6 [42]. At high resolution, the Arf6 and H-Ras proteins appeared to inhabit distinct domains of many of the larger endosomes, producing a beaded appearance in cross-section (Fig. 2D). The constitutively active H-Ras61L was also present on Arf6-WT-associated endosomes (data not shown). These results agree with the previous finding that endogenous Ras proteins can be found on endosomes regardless of their activation state [10]. Complex cellular changes that affect endocytosis accompany transformation caused by long-term expression of activated H-Ras61L [9]. To learn if these changes included altered H-Ras61L binding to Arf6-associated endosomes, Arf6 was expressed in a stable cell line harboring H-Ras61L. In the transformed cells, oncogenic H-Ras61L was found on endosomes coated with Arf6 (data not shown).

The results indicated that endosomes that contained Arf6 could bind H-Ras in both the GDP- and GTP-bound states. This data provides a complement for the earlier biochemical experiments that indicated that neither H-Ras-WT nor H-Ras61L nor EGF-stimulated H-Ras-GTP bound clathrin-dependent endosomes.

To examine if Arf6 activation changed the ability of H-Ras to bind these endosomes, an Arf6 protein that favors the GTP-bound form (Arf6-Q67L) was used. The Arf6-Q67L protein supports the early steps of endosome formation in a normal fashion, but the inability of the protein to hydrolyze its bound GTP prevents the nascent endosomes from further progress, leading to an endocytic cul-de-sac and formation of multiple, enlarged endosomes [29]. In cells co-transfected with Arf6-Q67L-GFP and H-Ras61L, the H-Ras61L protein brightly outlined the distinctive groups of large endosomes that were also positive for Arf6-Q67L-

GFP (Fig. 2E). Thus, H-Ras could also bind to endosomes in which the Arf6 protein was GTP-bound.

To further characterize these endosomes, cells were transfected with a hemagglutinin (HA)-tagged version of Arf6-Q67L-HA along with GFP-H-Ras61L so that Arf6-containing membranes could be immuno-isolated using HA antibody. On detergent-free fragments of Arf6-HA-containing membranes that were isolated with anti-HA antibody, GFP-H-Ras61L was present (Fig. 2F). A reverse experiment, using H-Ras-directed antibody capture, showed that Arf6-Q67L-GFP could also be detected on H-Ras-containing membrane fragments (Fig. 2G). In addition, a control experiment was performed, in which anti-HA antibody-directed membrane isolation was performed using cells that expressed H-Ras but that had no Arf6-HA to capture. The resulting sample contained no H-Ras (Fig. 2H). This showed that the presence of H-Ras in the sample required Arf6, and was not the result of incomplete membrane washing or non-specific binding of H-Ras to the HA antibody.

EGF and H-Ras activation on Arf6 endosomes

Given the constitutive nature of endocytosis via the Arf6-associated pathway an important issue was whether binding to these endosomes would result in rapid down regulation of H-Ras GTP binding. This question had not been answered by the previous experiment with H-Ras61L, because H-Ras61L GTP hydrolysis is impaired. This experiment required a cell to simultaneously express 3 proteins: Arf6, H-Ras-WT, and GFP-RBD (a GFP-tagged version of the Ras binding domain of Raf-1) to bind to and report the location of the GTP-bound form of H-Ras. To accomplish this with only two color filters, the activated Arf6-Q67L was used so that its presence could be detected by the distinctive clustering of endosomes it produced, rather than by staining. The triply transfected cells were then either

left untreated or were exposed to a low concentration of EGF for varying times before fixation and imaging.

In untreated cells, the GDP-bound H-Ras-WT protein outlined the plasma membrane and clustered endosomes as before, but the GFP-RBD did not, and was instead nearby in the cytosol (Fig. 3A). This is the appropriate location for GFP-RBD in an unstimulated cell. After 5 minutes of exposure of the cells to EGF, GFP-RBD could now be seen both at the plasma membrane and on many H-Ras-WT-coated enlarged endosomes (Fig. 3B). Notably, within this short time frame, no significant binding of GFP-RBD to perinuclear membranes that might be Golgi was observed [43]. This indicated that H-Ras-WT could become GTP-bound at the plasma membrane and on Arf6-associated vesicles that were already internal or that entered the cell during the 5 minutes of EGF exposure.

Previous studies had shown that total H-Ras GTP binding remained elevated for several minutes after EGF stimulation, and returned to baseline within about one-half hour [11]. Similarly, in this experiment, after 10 minutes of exposure to EGF, GFP-RBD remained visible on both the plasma membrane and on many Arf6-Q67L vesicle membranes (Fig. 3C). Thus, during EGF stimulation, H-Ras continued to associate with, and retained GTP while bound to, Arf6 vesicles. There was no indication that endosomal H-Ras lost GTP more rapidly than H-Ras at the plasma membrane. After 30 minutes of EGF exposure, GFP-RBD binding to the Arf6 vesicles did decline, indicating that H-Ras GTP binding had dropped (data not shown). These results indicated that binding of H-Ras to vesicles of the Arf6-associated endocytic pathway is neither a method for rapid “down regulation” of H-Ras activity nor a mechanism to prolong H-Ras GTP binding.

Very little Raf-1 binds to H-Ras61L on Arf6 endosomes

The initial studies described above had indicated that although endosomes that contained EEA1 did not bind H-Ras, they did bind the H-Ras effector protein, Raf-1. The finding that GTP-bound H-Ras was present on Arf6-associated vesicles raised the question of whether some Raf-1 might also be present with H-Ras-GTP on Arf6-coated vesicles. It was necessary to use immunofluorescence to examine Arf6 endosomes specifically, rather than membrane immuno-isolation, because Arf6 is present on both the plasma membrane (where Raf-1 is known to associate with active H-Ras) and on endosomal membranes. Cells were co-transfected with DNAs for Arf6-Q67L (to generate visibly identifiable endosomes) and H-Ras61L, then were stained with antibodies for endogenous Raf-1. As before, the characteristic vesicles and clustered Arf6-Q67L endosomes were brightly rimmed with GFP-H-Ras61L (Fig. 4A). However, the Raf-1 antibody did not illuminate those clustered endosomes (Fig. 4B), and in only a few spots did endogenous Raf-1 and H-Ras61L colocalize on endosomes. (Fig. 4C) A second experiment examined whether endosomes that displayed transfected Arf6-Q67L-GFP could bind Raf-1. Once again, there was no alignment (Fig. 4D). These results suggested, unexpectedly, that endosomal H-Ras, although in the GTP-bound form, bound very little Raf-1. Further experiments are planned to learn if endosomal Raf-1 is completely separate from endosomal H-Ras-GTP.

H-Ras is present in the perinuclear endosomal recycling center

Although some H-Ras is present on Golgi membranes, recent studies have found that H-Ras persists in the perinuclear area despite clearing the H-Ras biosynthetic pathway by exposing cells to cycloheximide [7, 8]. Our own studies (see below) confirmed that even after prolonged interruption of biosynthesis, H-Ras was present (i.e., either retained or continuously replaced) on perinuclear membranes. The contribution of endosomal pathways,

and in particular the perinuclear endosomal recycling center (ERC), to the internal pool of H-Ras was investigated.

In order to specifically identify the ERC and to distinguish it from Golgi membranes, transferrin, a protein known to enter the ERC, was used. In cells pre-treated with cycloheximide for 6 hours, live-cell uptake of AlexaFluor-transferrin for 45 minutes at 37°C produced a strong perinuclear accumulation, along with numerous peripheral vesicles that were entering or recycling to the plasma membrane (Fig. 5A). In these cycloheximide-treated cells, the previously synthesized H-Ras61L that was located on internal membranes colocalized entirely with the perinuclear concentration of AlexaFluor-transferrin (Fig. 5A). Transfected H-RasWT also co-aligned well with the transferrin-marked ERC (data not shown). This indicated that the internal membranes populated by previously synthesized H-Ras were predominantly those of the ERC, and that, with this protocol, very little H-Ras was located on Golgi membranes. This data supported the recent report that H-Ras is associated with Rab11-positive vesicles in the recycling center [12]. Notably, at the periphery of the cell, the previously synthesized GFP-H-Ras61L was present in small vesicles that were separate from those that contained AlexaFluor-transferrin (Fig. 5B). This corroborated the results shown above, which indicated that H-Ras61L could bind vesicles that were separate from those that carried transferrin. The ERC region was too densely packed with vesicles to determine if the H-Ras-containing vesicles simply intermingled or actually merged with transferrin-containing vesicles. These results indicated that, in addition to endosomal membranes and membranes of the Golgi, H-Ras could distribute to a third type of internal membrane -- the membranes of the ERC.

KRas4B and ExtRas binding to Arf6 endosomes differs from H-Ras

The route of the outward, biosynthetic trafficking of the non-palmitoylated KRas4B protein is unknown, but appears to differ from the vesicular mechanism of H-Ras trafficking from the Golgi. We therefore examined if these two Ras proteins also differed in either endocytosis or recycling. Activated GFP-KRas4B-G12V protein was clearly seen on the plasma membrane and on Arf6-Q67L-enlarged endosomes (Fig. 6A). This result indicated that activated KRas4B could also bind Arf6-associated vesicles, and was not excluded from this pathway. However, in cells loaded with Alexa Fluor-transferrin (without Arf6-Q67L), activated KRas4B was not present in the ERC (Fig. 6B). Thus, KRas4B differed from H-Ras and did not bind either ERC or Golgi membranes.

To learn more about the properties that were responsible for the binding of H-Ras to Arf6 endosomes, a mutant H-Ras protein with C-terminal characteristics of both H-Ras (palmitates) and KRas4B (lysine residues), was examined [34]. This protein (ExtRas) is a mutant of H-Ras with a C-terminal extension of 6 lysines that act to prevent attachment of farnesyl. Because of its lack of farnesylation, ExtRas also fails to undergo proteolysis or methylation, and does not interact with ER or Golgi membranes where these modifications take place [34, 44]. In addition, ExtRas shows no evidence of binding to exocytic vesicles, but does associate with the plasma membrane and become palmitoylated [34]. Interestingly, ExtRas differed from H-Ras and KRas4B, and was not detectable on Arf6-WT endosomes (Fig. 6C). Furthermore, ExtRas61L did not colocalize with AlexaFluor-transferrin in the ERC (Fig. 6D). This was an important control because, although endocytosis via Arf6-associated vesicles can occur continuously, binding to these endosomes was neither universal nor inevitable for a palmitate-modified H-Ras protein.

The C-terminal domain of H-Ras or KRas4B proteins can target GFP to the Arf6 endocytic pathway

Because both GDP-bound and GTP-bound forms of H-Ras were found on Arf6-associated vesicles, it appeared that the Switch I and Switch II regions of the protein (which differ between the GDP vs GTP state) were not likely to be determinants of endocytosis. To study the structural requirements for endocytosis more directly, a GFP chimera, having only the last ten C-terminal residues of H-Ras was tested. Identical GFP-H-tail proteins have previously been shown to be correctly modified with farnesyl and palmitoyl lipids, and to be targeted to raft-like areas of the plasma membrane [20]. This GFP-H-tail protein also bound Arf6-Q67L endosomes (Fig. 7A). A second chimeric version of GFP, GFP-K-tail, with the last twenty residues of KRas4B, also bound Arf6-Q67L endosomes (Fig. 7B). These results mirrored the ability of the full-length H-Ras and KRas4B proteins to bind Arf6-Q67L endosomes. This indicated that the farnesylated C-termini of Ras proteins, with either palmitates or multiple lysine residues, were sufficient for binding to Arf6-coated endosomes.

Discussion

The studies here provide two notable findings—the absence of H-Ras from clathrin-dependent endosomes, and the identity of a second class of vesicles that do bind H-Ras. Cellular, growth factor stimulated, and oncogenic forms of H-Ras bind clathrin-independent endosomes that contain the Arf6 protein. These endosomes are separate from, and in addition to, vesicles of the biosynthetic pathway. Also, Arf6 endosomes provide a second mechanism of internal localization for a membrane-bound form of H-Ras that is distinct from the deacylation-controlled cycle between the cell surface, cytosol and internal membranes. Several other clathrin-independent endocytic routes are rapidly being defined [15, 24, 45,

46]. It will be important to learn if H-Ras also utilizes these pathways, or only the Arf6-associated route.

Both active and inactive forms of H-Ras were found on the Arf6 endosomes. The nucleotide-independence of H-Ras endosome binding is consistent with previous reports [10] and with the constitutive nature of Arf6-mediated endocytosis [25, 47, 48]. Internal relocation via deacylation/re-acylation also operates for both GDP- and GTP-bound forms of H-Ras [7, 8], but interestingly, palmitate turn-over is accelerated when H-Ras is in the GTP-bound form [36]. Arf6 endocytosis can also be regulated, and it remains to be seen how stimulation of Arf6-associated endocytosis [25, 40, 42] affects H-Ras-GTP that may be on those endosomes, and whether H-Ras signaling is influenced through this physical relocation.

Although the plasma membrane is widely viewed as the static, final destination for Ras proteins, this report adds support to the idea that the amount of Ras at the plasma membrane is dynamic and continuously adjustable, both through deacylation and by endocytosis.

Properties that influence binding of Ras proteins to Arf6-associated vesicles

Our results show that the seven, C-terminal residues of H-Ras, and the accompanying farnesyl, methyl, and palmitoyl groups, play an important role in endosome binding, just as they also do for association with Golgi membranes [6, 8]. The H-Ras protein that is found on Arf6-associated vesicles is likely to be palmitoylated, as it continues to bind strongly to membranes. However, palmitoylation cannot be the sole determinant for Arf6-based endocytosis, because the ExtRas protein is palmitoylated on C-terminal cysteines, and it failed to enter endosomes.

Because the GFP-H-tail protein resides in low-density (“raft”) membrane microdomains [49], its internalization probably takes place from these domains. The GFP-K-tail protein and

the full-length KRas4B-G12V protein, which reside outside of the raft-like domains [20] were also observed on Arf6-Q67L endosomes. These results are consistent with reports that Arf6-associated endosomes can internalize proteins from both types of domains [26].

The role of endosomes in H-Ras signaling

The separation of H-Ras from the clathrin-dependent pathway has special importance for interactions of H-Ras with its effector, Raf-1. Both in this work and in studies with insulin stimulated- [10], or angiotensin-stimulated cells [50], activated Raf-1 has been found primarily on endosomes derived from the clathrin-dependent pathway. The presence of Raf-1 on EEA1-containing endosomes is also consistent with the report that H-Ras-stimulated Raf-1 signaling is decreased by a dominant negative dynamin mutant [16]. Combined with the H-Ras results of these studies, these reports suggest that H-Ras-triggered Raf-1 signals can occur on classical early endosomes, without H-Ras being present. This possibility is also consistent with the findings that H-Ras:Raf interactions at the plasma membrane are actually quite brief [51].

In contrast, only a very small amount of Raf-1 was found on Arf6-associated vesicles that contained H-Ras-GTP. Furthermore, Arf6-associated endosomes also bind other signaling proteins, such as Src and Rac [26, 40]. This suggests that signals arising from endosomal H-Ras may differ from those of H-Ras on the plasma membrane. There is already precedent for selective association of nucleotide exchange factors (GEFs), effectors, and GTPase activating proteins (GAPs) with H-Ras that is present on Golgi membranes [1, 43].

These studies also reveal a new challenge for studies that wish to measure H-Ras signaling from internal sites -- the presence of H-Ras in the perinuclear recycling center. Because the endocytic recycling center is often embedded within or near the Golgi, it may be

necessary to distinguish H-Ras on ERC membranes from newly synthesized or re-acylated H-Ras on Golgi membranes [8].

Ras signaling pathways have previously been linked functionally with Arf6. H-Ras stimulates phosphatidylinositol 3-kinase activity, which leads to recruitment and activation of the Arf6 nucleotide exchange factor, ARNO [30]. Reciprocally, inhibition of the extracellular signal-regulated kinases (ERKs) that are downstream of Ras proteins, specifically impedes Arf6-mediated, but not clathrin-mediated, endocytosis [52]. Arf6 and H-Ras collaborate in vesicular transport, through co-stimulation of phospholipase D activity needed for vesicle budding [53]. Both Arf6 and H-Ras proteins are also central to the pinocytosis and membrane ruffling that are caused by growth factors. The studies reported here provide the physical foundation that will allow the reciprocal interactions of Arf6 and H-Ras, and the impact of those interactions on signaling, to be unraveled.

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Figure Legends

Figure 1 H-Ras is absent from early endocytic vesicles of the clathrin-dependent pathway.

All scale bars in this figure = 5 μ m.

- A) Merged images of adherent membrane fragment from sonicated cell expressing H-Ras61L. Cells were fixed, stained with antibodies to H-Ras (green) and clathrin heavy chain (red), and with fluorescent secondary antibodies, and were examined by indirect immunofluorescence microscopy.
- B) NIH 3T3 cells stably expressing H-RasWT cDNA were fixed and stained with antibodies to H-Ras (green) and EEA1 (red). Vertical z-stack images of each color were deconvolved and merged.
- C) Merged images of adherent membrane fragment from sonicated cell expressing H-Ras61L. Cells were fixed and stained directly with antibodies to H-Ras (green) and EEA1 (red) and with fluorescent secondary antibodies.
- D) NIH 3T3 cells stably expressing H-RasWT were treated with 10 ng/ml of EGF for 10 minutes before fixation and stained with antibodies to H-Ras (green) and EEA1 (red) and with fluorescent secondary antibodies. Vertical z-stack images of each color were deconvolved then merged. Inset is a higher magnification of the boxed area, to show separation of EEA-1 and H-Ras (arrowheads).
- E) Merged images of Alexa-Fluor transferrin (red, arrowheads) and GFP-H-Ras61L (green, arrows) in a cell transfected and grown for 18 hours, then treated with cycloheximide for 6 hours, chilled and exposed to transferrin, then warmed for 1

minute before fixation. Vertical z-stack images of each color were deconvolved before merging.

- F) Membranes from the post-nuclear supernatant (PNS) in NIH 3T3 cells stably expressing HRas-WT or H-Ras 61L were captured with EEA1 antibody (IP). Proteins were separated by SDS-PAGE and identified by immunoblotting with indicated antibodies. 95% of the sample was loaded in the IP lane, and 5% in the PNS lane.

Figure 2 H-Ras is present on endosomal membranes that contain MHC-I and Arf6

- A) HeLa cells were transfected with cDNA for GFP-H-Ras61L, incubated for 18 hours, and then treated with cycloheximide for 6 hours. During the last 30 minutes, anti-MHC-I monoclonal antibody was added to the culture medium. Cells were washed for 30 seconds with mild acid to remove surface-bound antibody, then were fixed, and stained with mouse secondary antibody (red). Vertical z-stack images of each color were deconvolved. Arrowheads indicate positions of several vesicles containing the internalized MHCI and GFP-HRas61L (green).
- B) HeLa cells were transfected with cDNA for Arf6-Q67L-GFP (green), incubated for 18 hours then treated with cycloheximide for 6 hours. During the last 30 minutes, anti-MHC-I was added to the culture medium. Cells were washed briefly with mild acid, fixed, incubated with mouse secondary antibody (red) to detect the anti-MHC-I, and imaged. Vertical z-stack images of each color were deconvolved and merged.
- C) Confocal image of transfected H-RasWT (red) on endosomes in cell expressing Arf6-WT-GFP (green). Scale bar = 10 μ m. Cells were stained with appropriate primary and secondary antibodies and were examined by indirect immunofluorescence microscopy.

- (D) Enlargement of merged H-Ras (red) and GFP (green) images, from another cell, to show the beaded appearance (arrow) of Arf6 and H-Ras on the endosome membrane. Scale bar = 5 μ m.
- E) Confocal images of a cell transfected with cDNAs encoding activated H-Ras61L (red) and Arf6-Q67L-GFP (green). Scale bar = 10 μ m.
- F) Cells were co-transfected with cDNAs encoding GFP-H-Ras61L and Arf6-Q67L-HA. Membrane fragments from the post-nuclear supernatant (PNS) were captured with anti-HA antibody (IP). Proteins present in the membranes were separated by SDS-PAGE and detected on immunoblots with anti-HA (for Arf6) and anti-GFP (for GFP-H-Ras). Asterisk (*) shows position of antibody heavy chain from the antibody used for immunoprecipitation.
- G) Cells stably expressing H-Ras61L were transfected with cDNA encoding Arf6-Q67L-GFP. Membranes from the post-nuclear supernatant (PNS) were captured with anti-Ras antibody (IP). Proteins present in the membranes were separated by SDS-PAGE and detected on immunoblots with anti-Ras and anti-GFP (for Arf-GFP) antibodies.
- H) Anti-HA antibody isolation performed on membrane fragments from the post-nuclear supernatant (PNS) of cells expressing H-Ras-61L?-GFP? (IP). Proteins present in the sample were separated by SDS-PAGE and detected on immunoblots with anti-GFP (for GFP-H-Ras). Asterisk (*) shows position of antibody heavy chain from the antibody used for immunoprecipitation.

Figure 3 EGF exposure results in GFP-RBD recruitment to enlarged endosomes in cells expressing H-RasWT and Arf6-Q67L

Confocal images of cells transfected with cDNAs encoding Arf6-Q67L-HA (unstained), cellular H-Ras (red), and the Ras-binding domain from Raf kinase (GFP-RBD, green)

- to detect GTP-bound H-Ras. At 24 hours after transfection, cells were exposed to EGF for the indicated times, fixed, and stained with anti-H-Ras. Arrows indicate H-Ras or GFP-RBD on plasma membrane; arrowheads indicate H-Ras or GFP-RBD on enlarged endosomes that result from expression of Arf6-Q67L. Scale bars = 10 μ m.
- A) Control cell that was transfected with all three cDNAs, but not untreated with EGF.
 - B) Transfected cell treated with 5 ng/ml EGF for 5 minutes.
 - C) Transfected cell treated with 5 ng/ml EGF for 10 minutes. The chevron indicates lack of GFP-RBD on some endosomes after 10 minutes exposure to EGF.

Figure 4 Raf-1 is present on Arf6-containing endosomes

All scale bars in this figure = 10 μ m.

- A) Merged images of H-Ras61L-GFP (green) and endogenous Raf-1 (red) in a cell also expressing Arf6-Q67L-HA (unstained). Cells were transfected, then fixed and stained with antibody to Raf-1. Arrowheads indicate points where Raf-1 is present on rim of enlarged endosomes with H-Ras61L. Vertical z-stack images of each color were deconvolved then merged.
- B) NIH 3T3 cells stably expressing H Ras61L were transfected with cDNAs for Arf6-Q67L-GFP (green) and Raf1- myc (red). Cells were fixed and stained with anti-myc antibody. Vertical Z-stack images of each color were deconvolved.
- C) Cells stably expressing GFP-H-Ras (green) were transfected with cDNAs for Raf1-myc (red) and the cellular form Arf6-WT-HA (unstained), fixed, and stained with anti-myc antibody. Vertical Z-stack images of each color were deconvolved. Arrowheads indicate endosomes with GFP-H-Ras61L and Raf1-myc.

D) Cells were transfected with cDNAs for Arf6-Q67L-GFP (green) and Raf1-myc (red), then were fixed and stained with myc antibody. Vertical Z-stack images of each color were deconvolved. Raf-myc is absent (cheveron) on Arf6-coated endosomes indicated in the right panel (arrowhead)

Figure 5 H-Ras is present in the perinuclear endosomal recycling center

All scale bars in this figure = 10 μ m.

- A) Confocal images of cell transfected with cDNA for H-Ras61L, treated with cycloheximide for 6 hr, exposed to AlexaFluor-transferrin (red) for 45 min, then fixed and stained with antibody against H-Ras (green). Arrowheads indicate perinuclear accumulation of H-Ras61L or transferrin.
- B) Cell treated as in (A). Boxed area is enlarged at right to show separate vesicles (arrowheads) of red and green.

Figure 6 Endocytosis of KRas4B and ExtRas differs from H-Ras

All scale bars in this figure = 10 μ m.

- A) Confocal images of cell expressing GFP-KRas4B-12V (green) and Arf6-Q67L-HA (red) on enlarged endosomes (arrowheads). Cell was transfected with cDNAs for GFP-KRas4B-12V and Arf6-Q67L-HA, fixed, and stained with anti-HA antibody.
- B) Merged confocal images of a cell expressing GFP-KRas4B-12V (green) and exposed to AlexaFluor-transferrin (red) for 45 minutes, then fixed. Chevron indicates perinuclear accumulation of transferrin, but not KRas4B in the endosomal recycling center.
- C) Confocal images showing ExtRasWT (red) is present on plasma membrane but not on Arf6-WT-GFP enlarged endosomes (green). Chevrons in left panel indicate absence

of ExtRas on Arf6-WT-coated endosomes (arrowheads) that can be seen in right panel.

- D) Merged confocal images of a cell expressing ExtRas61L (green) after exposure to AlexaFluor-transferrin (red) for 45 minutes. Cell then was fixed and stained with antibody to H-Ras. Chevron indicates perinuclear accumulation of transferrin, but not ExtRas61L, in the endosomal recycling center.

Figure 7 C-terminal residues of H-Ras or KRas4B proteins can target GFP to the Arf6 endocytic pathway

All scale bars in this figure = 10 μ m.

- A) Merged images of a cell expressing GFP-H-tail (green) and Arf6-Q67L-HA (red).
B) Merged images of GFP-K-tail (green) on Arf6-Q67L-HA (red) endosomes.

Figures

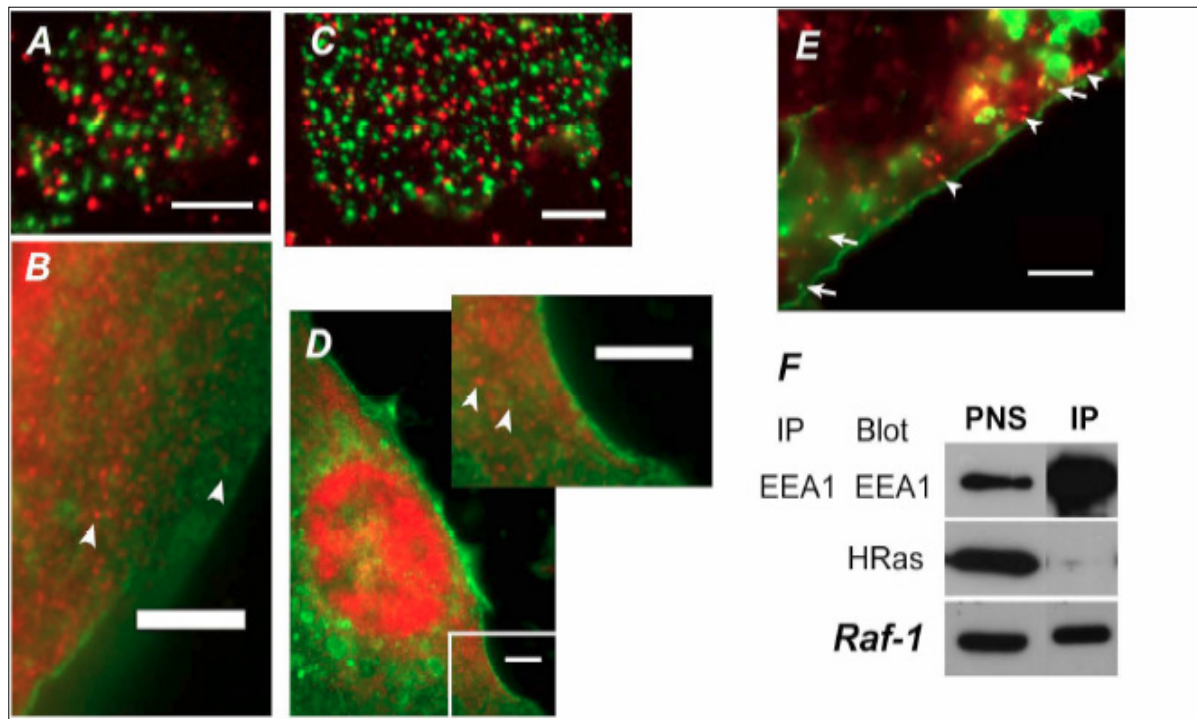


Figure 1 H-Ras is absent from early endocytic vesicles of the clathrin-dependent pathway

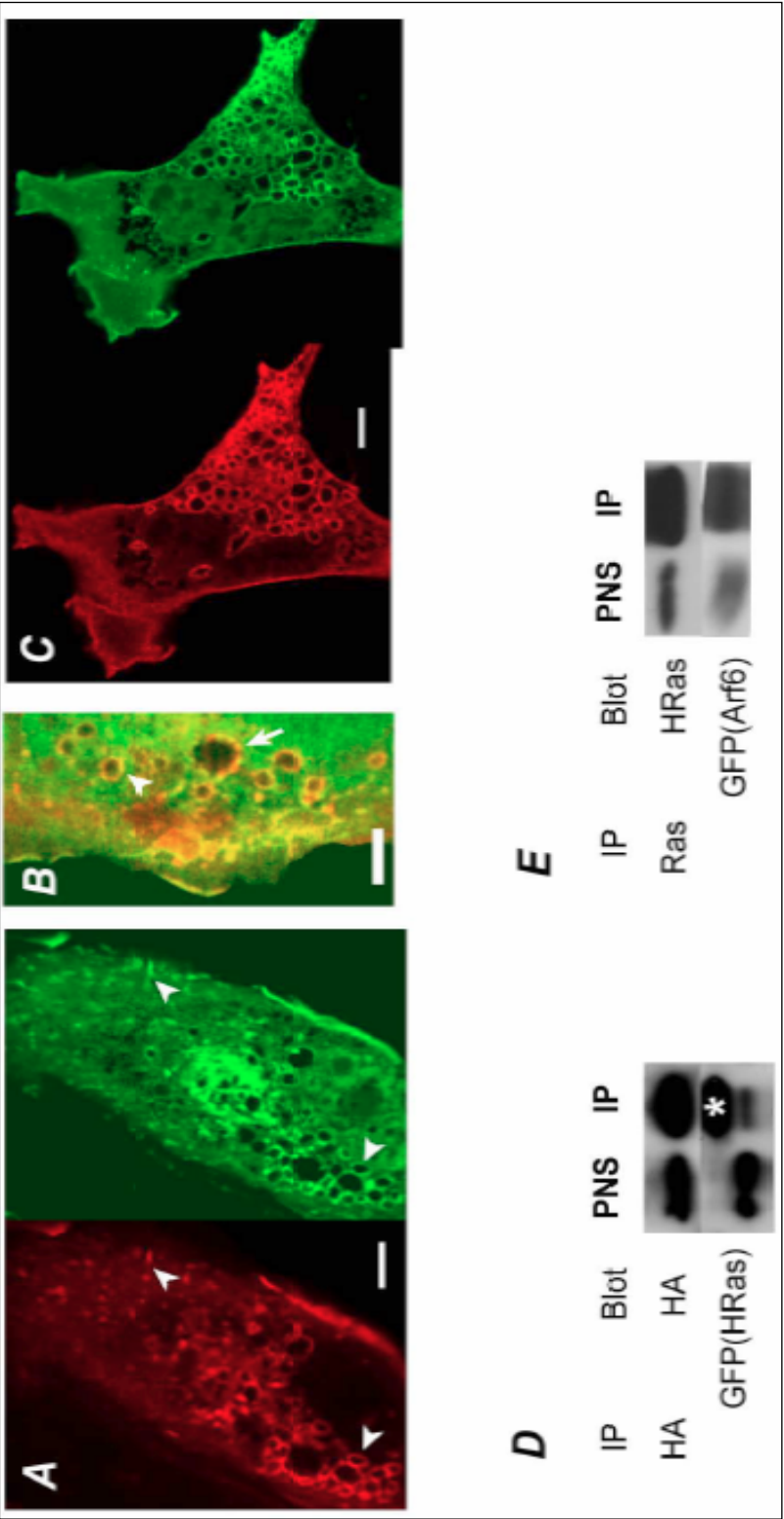


Figure 2 H-Ras is present on endosomal membranes that contain MHC-I and Arf6

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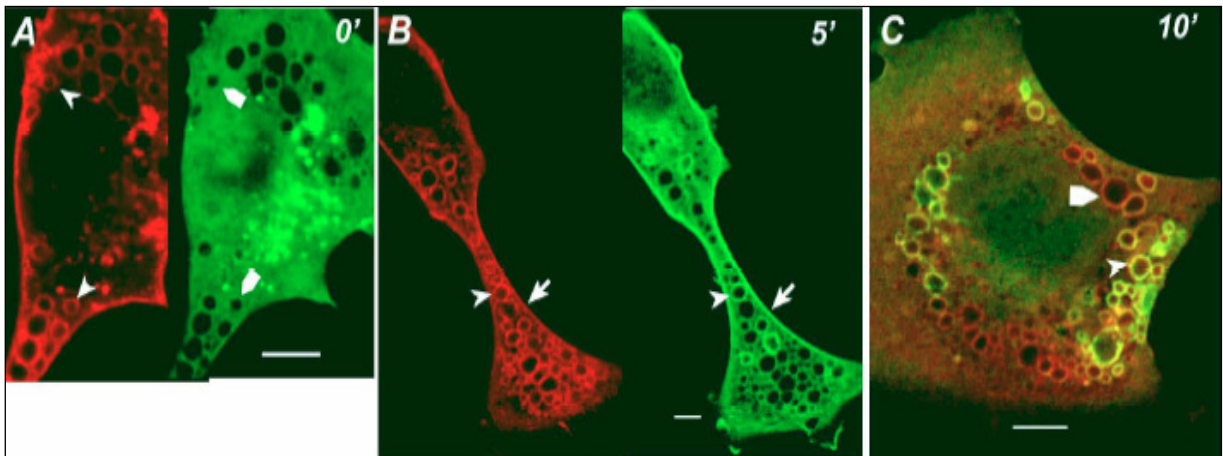


Figure 3 EGF exposure results in GFP-RBD recruitment to enlarged endosomes in cells expressing H-RasWT and Arf6-Q67L

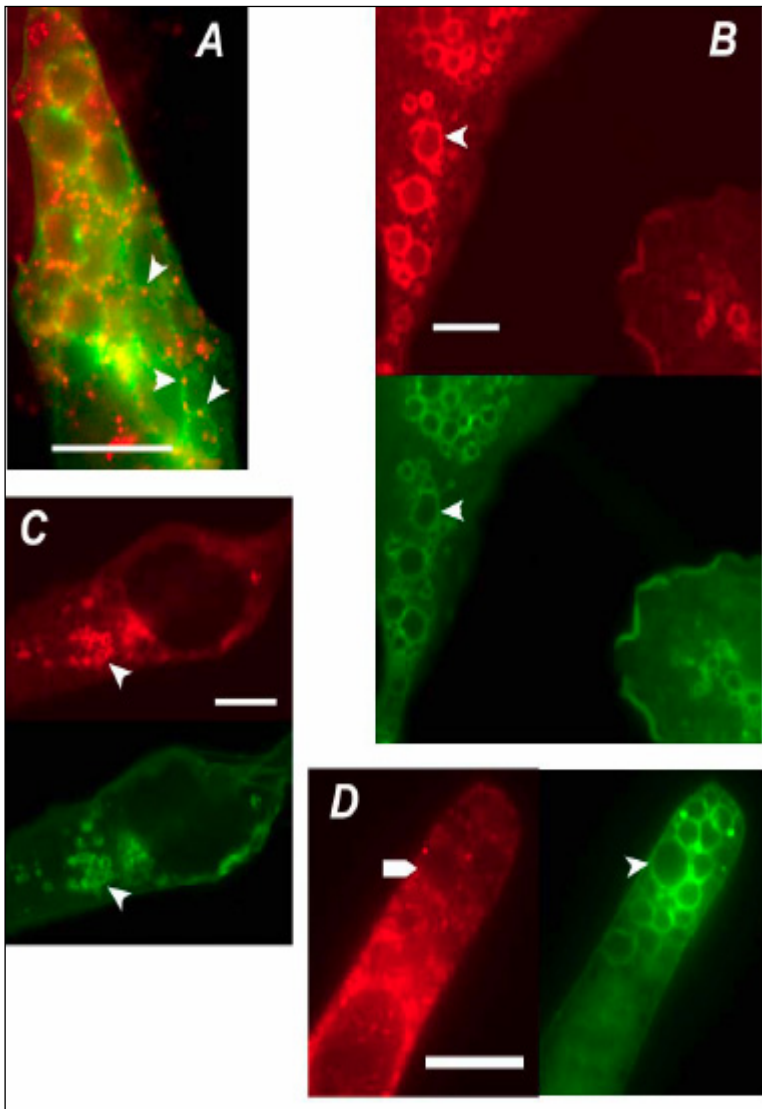


Figure 4 Raf-1 is present on Arf6-containing endosomes

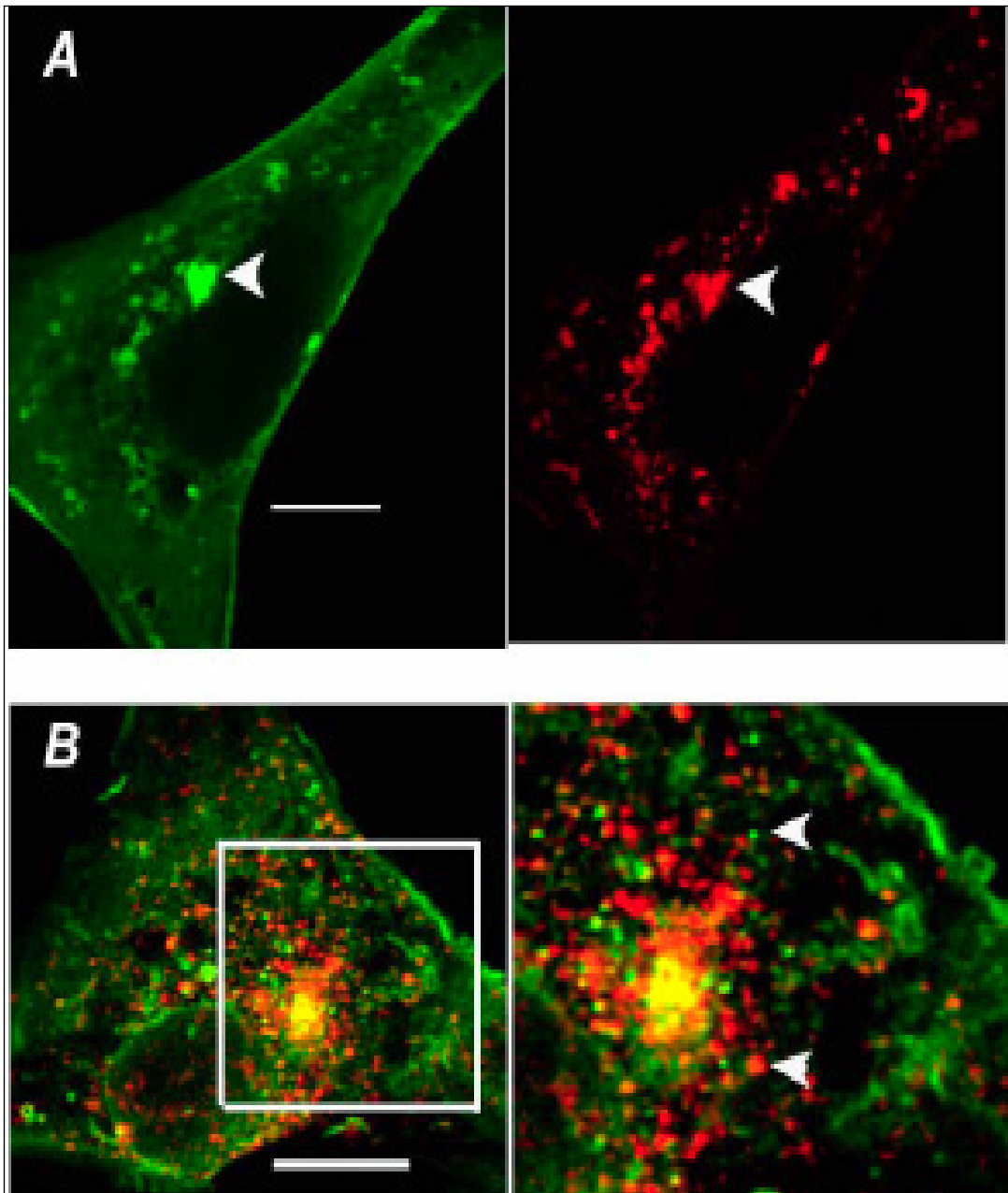


Figure 5 H-Ras is present in the perinuclear endosomal recycling center

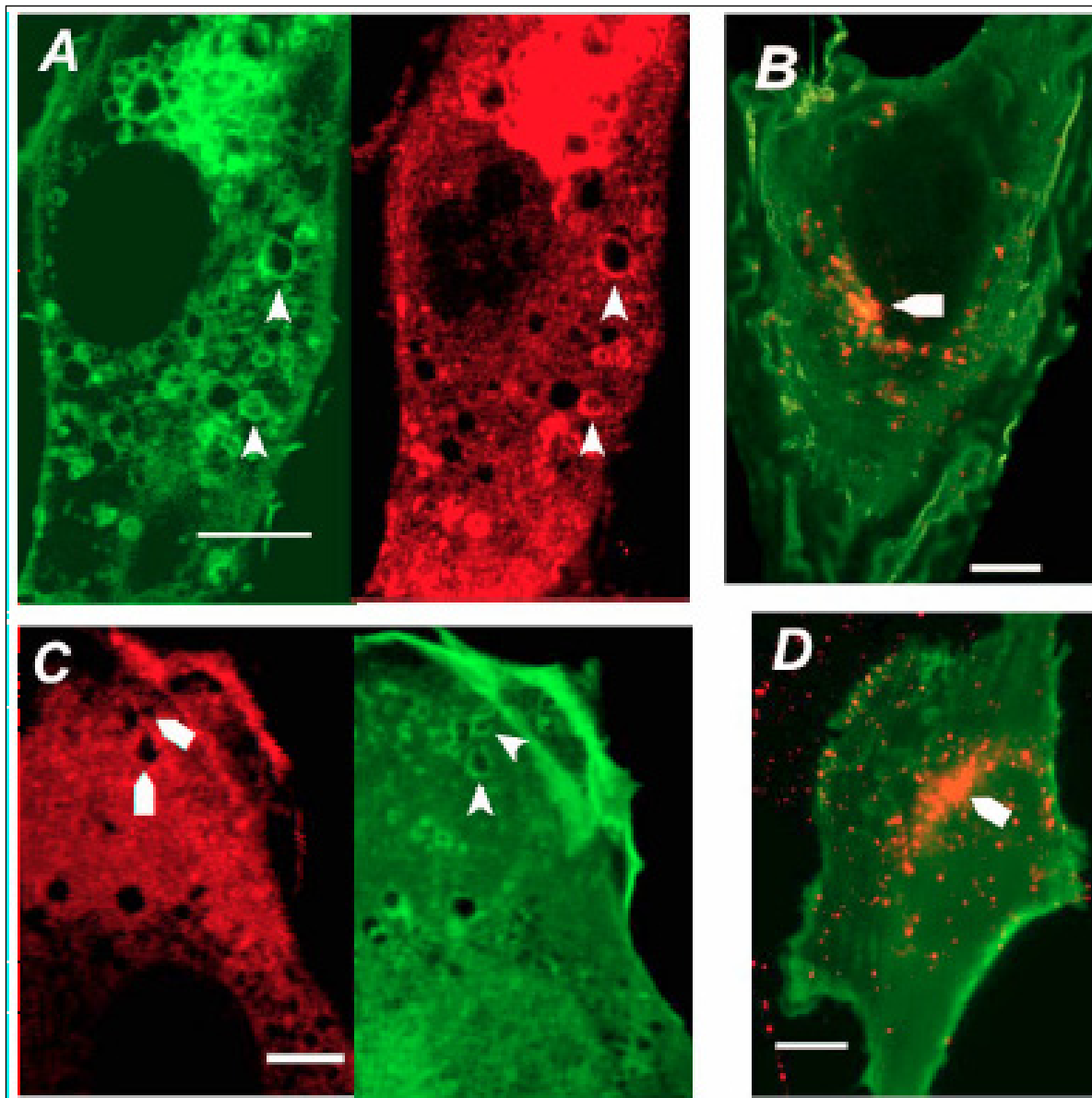


Figure 6 Endocytosis of KRas4B and ExtRas differs from H-Ras

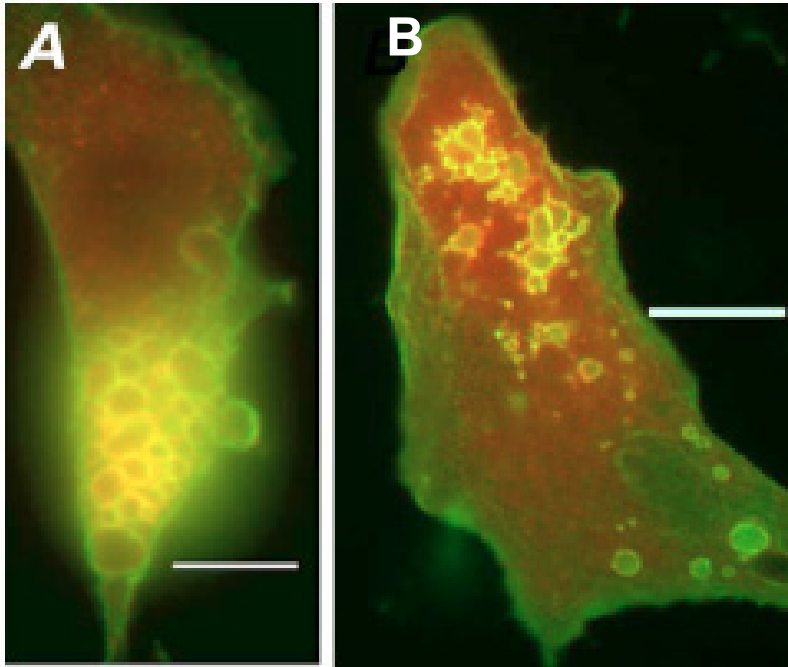


Figure 7 C-terminal residues of H-Ras or KRas4B proteins can target GFP to the Arf6 endocytic pathway

Chapter 5

General Conclusions

This dissertation describes intracellular trafficking and signal transduction of Ras, a member of a small family of G proteins. Ras is activated when it is bound to GTP and deactivated when it is bound to GDP. [1, 2] Within the Ras family, there are four major isoforms of Ras—NRas, KRas A, KRas B and HRas. Although more than 85% of the isoforms are conserved with one another, the isoforms differ from each other in their hypervariable region. **(Figure 1)** [3]

At the molecular level, by activating its downstream signaling effectors such as RAF and PI3K, **(Figure 2)** [4] Ras functions as a molecular switch that conveys information from the extracellular environment to the intracellular environment.

At a cellular level, Ras activation results in cell proliferation, differentiation, and apoptosis depending on the cell type in which Ras is activated. [2]

Ras posttranslational modifications

Before Ras can function as a molecular switch, it must first be posttranslationally modified at the carboxy terminus. The four amino acids at the carboxy terminus of Ras are often described as the CAAX box (*C* = cysteine, *AA* = aliphatic amino acid, *X* = any amino acid). Ras posttranslational modifications begin when the cysteine of the CAAX box gains a farnesyl lipid group. [5] For KRas, no additional modification is required, but for NRas and HRas, further modifications are needed. For example, NRas obtains a single palmitate group on cysteine 184 just upstream of the CAAX box, whereas HRas gains palmitates on both cysteine 181 and cysteine 184. **(Figure 3)** Once these modification processes are complete, Ras traffics to the plasma membrane and activates its signal transduction cascade that can

cause cells to proliferate, differentiate, or die. When the signal transduction cascade is constitutively activated, cells can become cancerous.

The results of the experiments conducted as part of the research reported in the three, related articles (chapter 2, 3, and 4 of this dissertation) seem to show that Ras can use the plasma membrane and membranes of subcellular organelles as signaling platforms. Recent studies demonstrate that signal transduction cascades initiated from endomembranes are distinct from one another and from the signal transduction cascade initiated from the plasma membrane. This dissertation focuses primarily on trafficking and signaling of HRas even though various isoforms of Ras and Ras mutants were used in the experiments reported here.

Ras intracellular trafficking

Typically, following the posttranslational modification, NRas and HRas traffic via vesicular exocytosis to the plasma membrane. This mode of trafficking begins at the Endoplasmic Reticulum (ER) where Ras proteins aggregate toward the ER exit sites. ER exit sites are identifiable by accumulations of Coat Protein Complex II (COP II) that are activated by ARF1 and Sar1. Once Ras is tethered on the outside of the vesicles, Ras passes through the ERGIC, an intermediate, vesiculo-tubular structure, before arriving at the Golgi. [6, 7] While some ER-resident proteins have an opportunity to return to the ER via Coat Protein Complex I (COP I) coated vesicles, most Ras proteins will continue to travel toward the plasma membrane via vesicles and microtubules. [8-10] (**Figure 4**)

Although vesicular exocytosis has been described as its primary trafficking mechanism, our own findings suggests that Ras traffic via a mechanism that is both Golgi- and vesicle-independent. While both traditional and the alternative trafficking mechanisms seem to

function simultaneously, the majority of the HRas proteins show a strong preference toward the alternative trafficking mechanism.

After Ras activates its signal transduction cascade from the plasma membrane, it can either be transiently removed from the plasma membrane by losing its palmitate groups or Ras can be more permanently removed by endocytosis. [11-13] There are several different vesicular endocytosis mechanisms but Ras typically utilizes a clathrin-independent, ARF6-mediated endocytosis. [14, 15] (**Figure 5**)

Summary of the Studies

With the general objective of understanding HRas intracellular trafficking and signal transduction cascades in mind, the three publications in this dissertation report examinations of HRas endocytosis, exocytosis, and amino acid conservation. These publications are summarized here.

HRas does not need COP I- or COP II-dependent vesicular transport to reach the plasma membrane

This manuscript focuses on HRas exocytosis from the ER to the plasma membrane.

When HRas was transiently expressed in cells, many did not show the perinuclear accumulation that is consistent with Golgi. This was particularly noticeable in HRas that were not attached to GFP. Traditional belief dictates that once HRas has been posttranslationally modified, it will pass through the ER, then ERGIC, and then the Golgi before reaching the plasma membrane via vesicles and microtubule tracks. However, in the experiments reported here, the majority of HRas did not accumulate in the Golgi, a finding that opens up the possibility of alternative trafficking mechanisms for HRas that are not dependent on the Golgi or vesicles.

The results seem to indicate that there is, indeed, a Golgi-independent trafficking mechanism that is Brefeldin A-insensitive and Sar1-dominant-negative insensitive. Brefeldin A (BFA) is a pharmacological reagent that blocks the COP I complex of proteins from aggregating, so if exposure to BFA is prolonged, all of the Golgi vesicles retrograde to ER and the Golgi eventually disappears. Neither interruption of outward vesicular movement with nocodazole nor ablation of COPI or COPII vesicles provided any evidence of non-traditional vesicles that might carry H-Ras to the plasma membrane. Thus, the data currently suggest that this non-classical pathway is non-vesicular. A similar mechanism also occurs when Sar1 dominant negative is expressed. Nocodazole, a pharmacological reagent that hinders the polymerization/depolymerization cycle of microtubules, was used to verify that the Golgi-independent trafficking mechanism is also vesicle-independent.

Further examination revealed that the Golgi-independent mechanism is also nocodazole insensitive, implying that the Golgi-independent trafficking mechanism is also microtubule-independent. In addition, because vesicles travel along the microtubule tracks, it can also be assumed that the Golgi-independent trafficking is non-vesicular. In addition, we demonstrated that the Golgi-independent trafficking pathway begins at the ER. Newly synthesized HRas located in the ERGIC were not released into the cytosol and trafficked to the plasma membrane in the presence of BFA and nocodazole. This suggests that HRas proteins in ERGIC are already committed to a vesicular trafficking mechanism. If HRas is somewhere before ERGIC, this place is mostly likely to be ER. In terms of their trafficking behavior, there were no observable differences between the constitutively active form of HRas and cellular form of HRas, which suggests that the activational state of HRas does not play a role in HRas trafficking.

Golgi-independent trafficking requires conservation of the HRas hypervariable region

This manuscript discusses the properties of HRas that are needed for HRas to utilize the non-vesicular, Golgi-independent trafficking mechanism by first establishing that the non-vesicular trafficking mechanism is available in various types of polarized epithelial cells and then showing that the presence of a GFP tag changes normal trafficking preferences for HRas. In Chapter 2 we reported that the 10 terminal amino acids of HRas are sufficient to enable the use of the alternative trafficking mechanism to reach the plasma membrane. In Chapter 3 we investigated whether palmitoylation is a key property needed in order to utilize non-vesicular trafficking. Mono-palmitoylated HRas mutants with substitutions at either position 181 or 184 reached the plasma membrane with ease, but used a vesicular trafficking mechanism.

This manuscript also discusses how non-palmitoylated residues in the HRas C-terminus are important for protein trafficking and localization. When an HRas mutant that retained all of the posttranslational modification sites but the residues in between the two palmitates were mutated (HRas CDQCECVLS), HRas was unable to reach the plasma membrane.

The residues that are far upstream of the CAAX box are also important for HRas trafficking. In delta 17 and delta 19 mutants, alanine substitutions were made for the residues 173–181 and 166–172 respectively. These mutants, like HRas C181S and HRas C184S, were able to reach the plasma membrane, but they did not localize to the plasma membrane when cells were treated with either BFA or nocodazole. Taken together these results show that every residue within the HRas hypervariable region plays a role in either HRas trafficking or membrane localization.

Internalization of HRas via ARF6-associated endosomal pathway

This manuscript reports on an investigation of the mechanisms by which HRas and KRas are removed from the plasma membrane and trafficked to the Endosomal Recycling Center (ERC). Although there are many different types of endocytosis, traditional thought has been that HRas and KRas endocytosis occurs predominantly through clathrin-coated vesicles.

In this study, however, Ras endocytosis was mediated by ADP Ribosylation Factor 6 (ARF 6 mediated) instead of being clathrin mediated. This study also suggests that the activation state of Ras does not affect ARF6-mediated Ras endocytosis, but that Ras located on ARF6 endosomes can be activated by one of its upstream regulators, Epidermal Growth Factor (EGF).

When stimulated with EGF, Ras actively localizes to ARF6-coated vesicles and recruits Ras Binding Domain of RAF (GFP-RBD). However, a full-length RAF was never visualized with HRas on ARF6 coated vesicles, which indicates that Ras is unable to complete its signal transduction while in transit through the Mitogen Activated Protein Kinase (MAPK) pathway.

We also examined properties of Ras to understand the requirements for Ras to use the ARF6-mediated endocytosis pathway. Our study suggests that both HRas and KRas are able to use the ARF6-coated vesicles for endocytosis. Furthermore, the 10 terminal amino acids of HRas and the 10 terminal amino acids of KRas 4B each contain sufficient localization signals to locate green fluorescence protein (GFP) to vesicular membranes. In other words, the cysteines for palmitoylation of HRas and the polylysine residues of KRas are localization signals. An interesting finding is that when these two are cut and ligated, they form HRas

Ext, which does not use ARF6 vesicles for endocytosis, which suggests that farnesylation of cysteine 186 at the CAAX box serves as a trafficking signal during endocytosis.

Ras in transit on ARF6 vesicles might be able to activate a signal transduction cascade, but apparently would not be able to complete the signal transduction and elicit a biological response such as cell proliferation.

Regardless of the endocytosis mechanism, ERC, a circular structure embedded within the Golgi, appears to be the final destination for most inward-bound vesicles. Both KRas 4B and HRas, but not HRas Ext, were observed in the ERC. HRas Ext lacks the farnesylation cysteine, which implies that farnesylation is an important property that dictates Ras endocytosis and recycling.

Biological Implications

In United States, cancer is the second most common cause of death, and each year thousands of new cases are diagnosed. It has been estimated that up to 30% of all cancers are caused by mutations in Ras. [16] At a cellular level, Ras activation causes cell proliferation, differentiation, and apoptosis. The specificity of the cellular response depends on the activated Ras isoform and tissue type. [3] Until recently, it has been thought that the plasma membrane is the only platform from which Ras can activate its signal transduction. Now, however, research results presented here and elsewhere suggests that Ras can signal from various endomembrane platforms which differ from those that are activated at the plasma membrane.

Recent advances in medical science and technology have yielded many new approaches to cancer therapies, particularly with regard to the ability of Ras to complete signal transduction cascades during endocytosis and in terms of the ability of Ras to complete

exocytosis both without the need for COP I or COP II and in a Golgi- and vesicle-independent manner. These findings suggest possibilities for increasing the effectiveness of those cancer therapies, especially in devising novel therapies for cancers that are driven by understanding and exploiting Ras lipid modifications, vesicular trafficking to the plasma membrane, and signal transduction cascades.

Suggestions for Future Research

The possibilities for future research based on the findings reported here can be considered in two categories, experiments that would support the research through control groups and experiments that would extend the research into new areas.

Additional control experiments

In this section, I suggest the following six possibilities for repeating the research reported here to enhance the robustness of the research.

- Would delta 17 and delta 19 alanine substitutions change the overall Ras-mediated biological responses?
 - How does dominant negative Ras also use Arf-6-mediated endocytosis?
 - Do Ras accumulate on the ARF6 vesicles when the ERK signaling cascade is inhibited?
 - Does palmitoyl transferase recognize the CDQCE mutant as Ras and attach palmitates?
 - What effect does the overall charges of amino acids have on Ras trafficking?
- Specifically, because DQ–E amino acid substitutions give Ras proteins an overall negative charge, would a mutant with a neutral charge change trafficking or membrane localization behavior?

- Are there differences in NRas trafficking patterns as compared with HRas trafficking patterns? Because palmitoylation is the driving force behind determining Ras trafficking behavior, and despite the fact that C184S HRas mimics NRas, do HRas and NRas differ in their hypervariable regions, and, if so, how?

Experiments that extend the research

The following seven research questions are by no means inclusive, but seem like logical routes toward extend our understanding of Ras trafficking and signal transduction.

- What are the possibilities for Ras non-vesicular endocytosis?
- Does NRas use Arf-6 vesicles in endocytosis?
- What is the exact trafficking mechanism behind the Ras acylation cycle?
- To what extent is the Ras hypervariable region conservation required during endocytosis?
- What is the mechanism behind KRas trafficking and how does it compare with HRas Golgi-independent exocytosis?
- Can Ras form dimers and oligomers with itself or with chaperones and/or escorts during exocytosis and endocytosis?
- What, if any, roles do the conserved domains of Ras play intracellular trafficking and signal transduction?

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Figure Legends

Figure 1 Ras domains

Structurally Ras proteins are divided into conserved domains and hypervariable regions.

The conserved domain begins at the N-terminus and extends to residue 165. This domain is well conserved all Ras isoforms. Within the conserved domain there are two switch regions (switch 1 and switch 2) where GTP/GDP exchange occurs.

Mutations in the switch regions can result in constitutively active or dominant negative mutants. The hypervariable region begins at the residue 166 and ends at the C-terminus. This region is posttranslationally modified with lipid groups. KRas 4 B acquires a farnesyl group at the CAAX box, whereas KRas A and NRas acquire an additional palmitate just upstream. HRas acquires two palmitates in addition to the farnesyl.

Figure 2 Ras-mediated MAPK signal transduction pathway

The best studied Ras-mediated signal transduction is the Mitogen Activating Protein

Kinase Pathway (MAPK). To begin the signal transduction cascade by activating Ras, a GEF exchanges a GDP for a GTP, then the Ras downstream effectors RAF, MEK and ERK activated by addition of phosphate groups. Activation of this signal cascade leads to cell proliferation, differentiation, and apoptosis. After initiating the MAPK signal transduction pathway, Ras becomes inactive by exchanging its GTP for a GDP.

This exchange is facilitated by GAP enzymes.

Figure 3 HRas C-terminus modifications

A newly synthesized HRas must be posttranslationally modified at the C-terminus before acting as a molecular switch. HRas posttranslational modification begins with the

addition of a farnesyl group at the C of the CAAX box by the farnesyl transferase. Then the —AAX of the CAAX box is cleaved by the Ras Converting Enzyme 1 (RCE 1), and the newly exposed C-terminus is methylated by the Isoprenyl cysteine Carboxy Methyl Transferase (Icmt). Finally, two additional palmitates are added to the upstream cysteines by palmitoyl transferase and complete HRas C-terminus modifications.

Figure 4 Ras exocytosis

A newly made Ras protein is located in the cytosol. Ras posttranslational modification begins with addition of a farnesyl group by the farnesyl transferase. This lipid modification kinetically traps HRas on the cytosolic surface of the ER membrane. Here the CAAX box is cleaved and two palmitates are added, so HRas posttranslational modification is complete. A mature HRas can move out to the plasma membrane by a vesicular trafficking mechanism, which includes passage through ERGIC and Golgi. Ras can also traffic to the plasma membrane directly from ER in a non-vesicular trafficking mechanism. KRas, which only contains a farnesyl group, localizes to the plasma membrane from ER in an unknown trafficking mechanism.

Figure 5 Endocytic vesicle trafficking

There are two major endocytic vesicle trafficking pathways—clathrin and non-clathrin.

In the clathrin trafficking pathway, clathrin coated vesicles are released from the plasma membrane and becomes an Early Endosome (EE). An EE can continue its trafficking into the cytosol and become a late endosome or it can merge with other vesicles and form a merged endosome. A merged endosome can either traffic to the

Endosomal Recycling Center (ERC) embedded in the Golgi or the merged endosome can traffic to the lysosomes along with late endosomes where the endosomal contents are degraded.

Figures

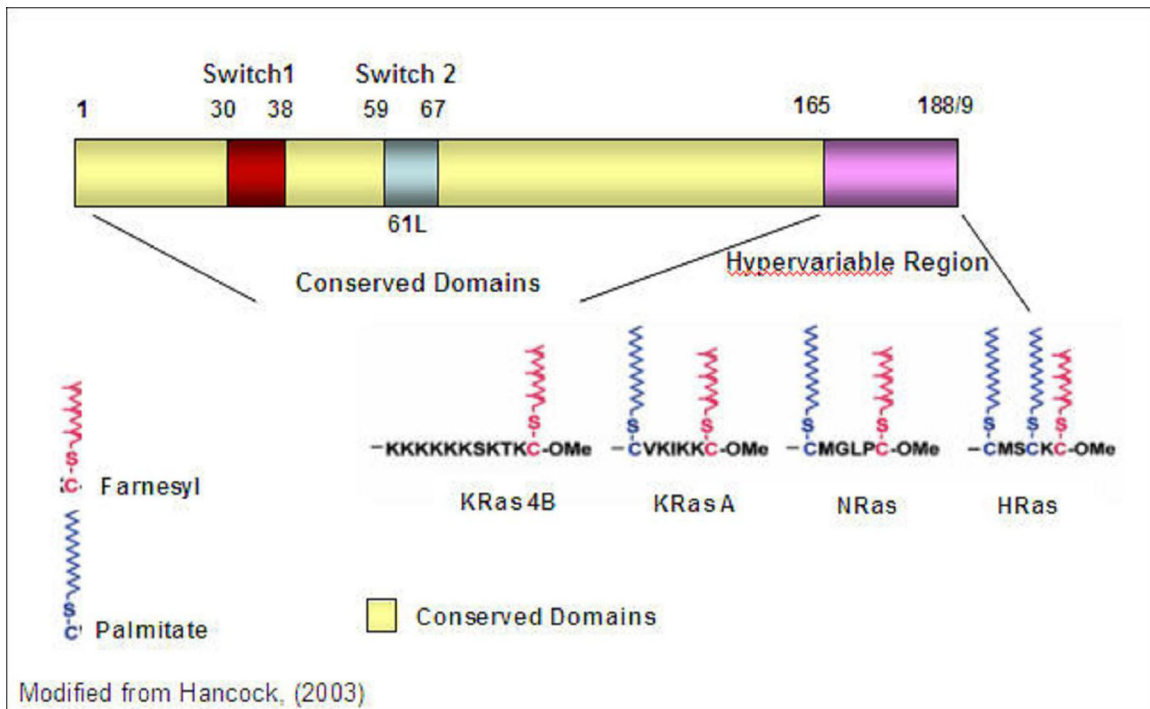


Figure 1 Ras domains

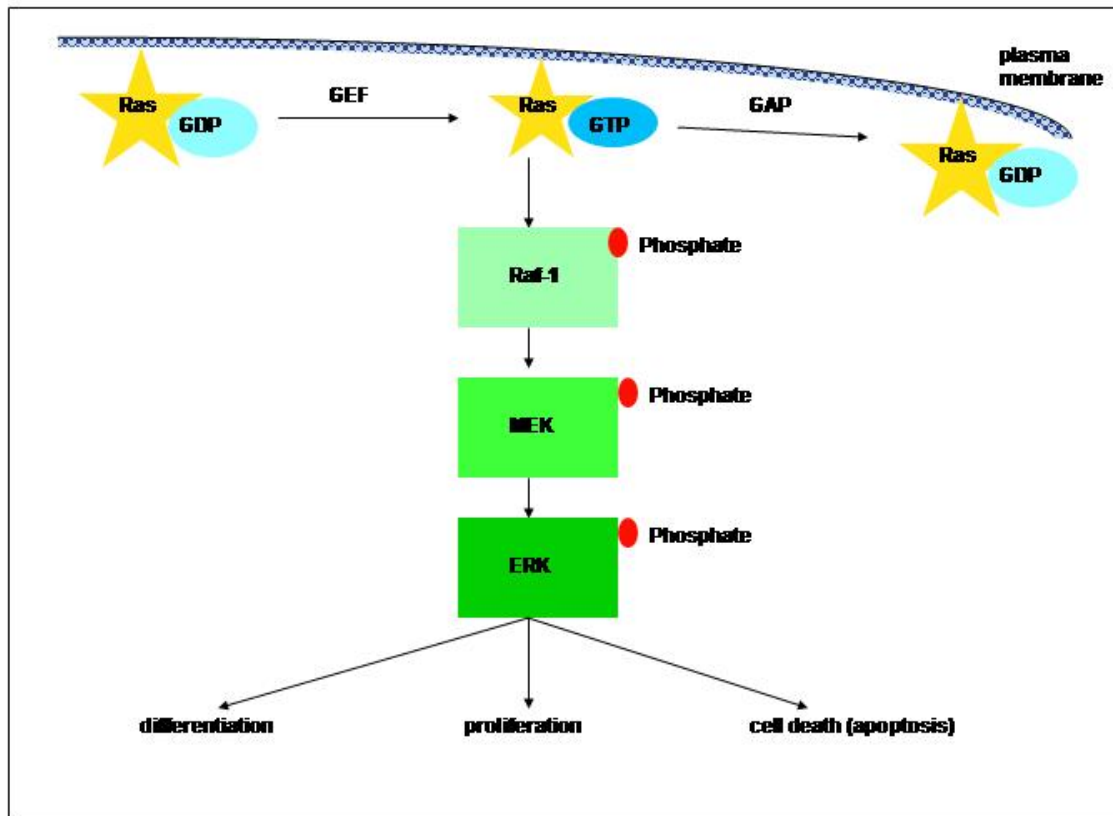


Figure 2 Ras-mediated MAPK signal transduction pathway

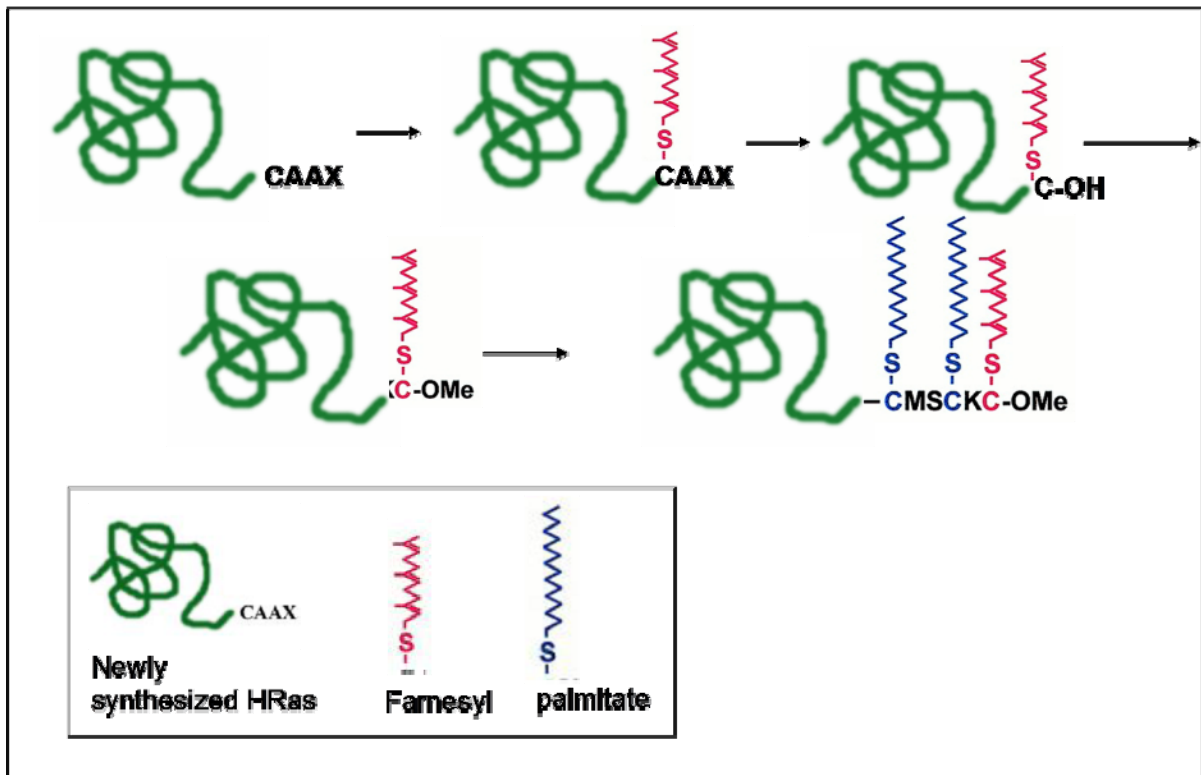


Figure 3 H-Ras C-terminus modifications

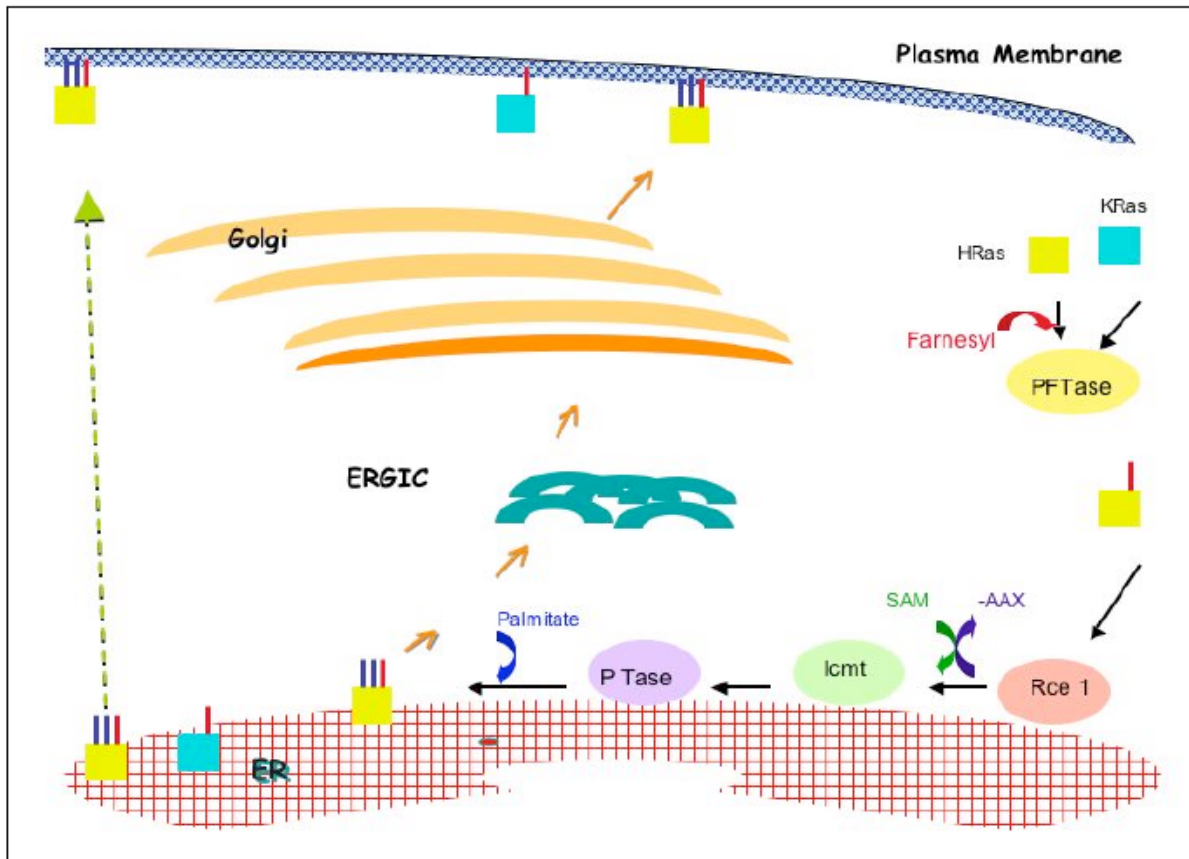


Figure 4 Ras exocytosis

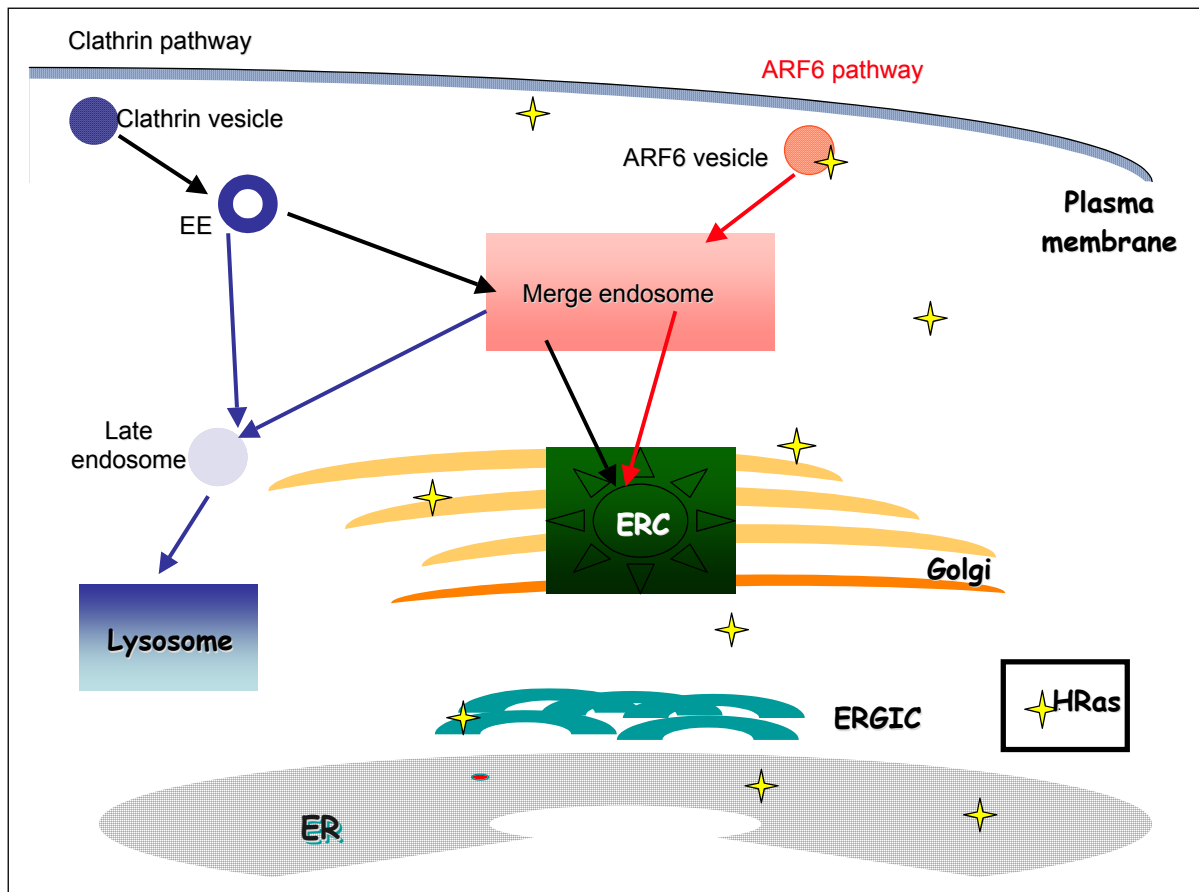


Figure 5 Ras endocytosis

Appendix

The CD-Rom that accompanies this dissertation contains a Windows Media Player movie file (.wmv) that shows GFP HRas Q61L granules moving intracellularly as described in “HRas Does Not Need COP I or COP II-Dependent Vesicular Transport to Reach the Plasma Membrane.” (dissertation Chapter 2)

These are the system requirements for playing this file:

Windows systems require Windows NT[®] 4.0 or later, Windows 98 or later and Windows Media Player 6.4 or later, Netscape Navigator 7.0 or later, or Microsoft Internet Explorer 5.0 or later.

Apple Macintosh systems require Apple Mac OS X or later, Windows Media Player for Mac OS X, or Internet explorer 5.2.2 or later.

For a free download of Windows Media Player for Mac or other platforms, visit

<http://www.microsoft.com/windows/windowsmedia/download>

Acknowledgements

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